EXPRESSION OF GLYCOPROTEINS IN WILD-TYPE AND VACCINE STRAINS OF VARICELLA ZOSTER VIRUS

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LING

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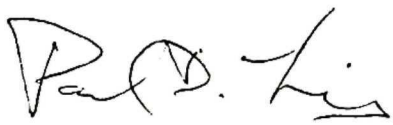
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ABSTRACT

Title of dissertation: Expression of Glycoproteins in Wild-Type and Vaccine Strains of Varicella Zoster Virus

Paul D. Ling, Doctor of Philosophy, 1990

Dissertation directed by: John Hay, Ph.D., Professor and Vice-chairman, Department of Microbiology

Characterization of varicella zoster virus(VZV) glycoprotein transcripts, polypeptides, and biologic properties is of major importance in understanding the growth cycle and pathogenisis of VZV. We have now characterized in detail those transcripts mapping to VZV glycoprotein IV (gpIV) and glycoprotein V (gpV). Northern blot, S1 nuclease and primer extension analyses, indicate that a major 1.65Kb transcript mapped to the gpIV open reading frame (ORF) and 1.95Kb and 2.5Kb transcripts mapped to the gpV ORF in a wild-type lab strain Scott. Likely TATA and CAT box motifs were located near the 5' termini and polyadenylation signals near the 3' termini of all these transcripts. Previously in our laboratory we observed that the live attenuated varicella vaccine strain, Oka, contained a 168bp deletion in the R2 repeat region found in the gpV ORF. The gpV transcripts in strain Oka were both smaller, 2.3Kb and 1.8Kb in size than

Scott, as well as >20-fold less in abundance. To test the mechanism for the deficient gpV transcription in Oka, we tested the R2 repeats from both Scott and Oka for enhancer activity but none was detected. Subsequently, the Scott and Oka promoters were tested for promoter activity in transient expression assays using gpV promoter/reporter gene fusions. The gpV promoters required viral transactivation with VZV, however, both Scott and Oka strains activated the gpV promoters similarly. The gpIV promoter was also studied in a similar manner described for gpV and was also shown to require VZV transactivation for activity. Additionally, it was shown that two VZV proteins ORF4 and ORF62 could stimimulate the gpIV promoter. The gpV polypeptide products were 95-105 kDa in size for strain Scott and displayed a patchy surface fluorescence in VZV-infected cells. Oka gpV polypeptides were slightly smaller in size, and accumulated to only 2% of the Scott gpV levels, correlating to the transcript levels. The gpV polypeptides were also glycosylated. These results imply that gpV may play a role in attenuation of the Oka virus. The gpIV polypeptide products ranged from 39-65 kDa in size, and were also glycosylated. Both of these proteins could elicit neutralizing antibodies in the absence of complement as tested in an *in vitro* plaque reduction assay. Finally, the gpV polypeptide product was unable to bind C3bi, and the gpIV polypeptide was not involved in Fc binding.

Expression of Glycoproteins in Wild-Type and Vaccine Strains of Varicella Zoster Virus

by

Paul D. Ling

Dissertation submitted to the Faculty of the Department of Microbiology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1990.

TO MY WIFE KAY

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Introduction

Varicella zoster virus (VZV) is a member of the *herpesviridae* family and the subfamily alphaherpesvirinae. The human alphaherpesviruses are comprised of VZV and herpes simplex viruses 1 and 2 (HSV-1 and HSV-2). Four other human herpesviruses are known to exist, Epstein-Barr virus (gammaherpesvirus), cytomegalovirus (betaherpesvirus), and human herpes viruses 6 and 7 (unclassified). While membership in the *herpesviridae* family is based on virion architecture, alphaherpesviruses are classified on the basis of variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily, but not exclusively, in sensory ganglia (Roizman et al., 1981; Roizman and Sears, 1990).

The Disease

Varicella zoster virus, as the name denotes, is the causative agent of two distinct clinical syndromes in humans. The first, varicella, which is commonly called chickenpox, results from a primary exposure to VZV, usually during childhood (Preblud et al., 1984). In developed countries like the U.S. almost everyone has been infected by adulthood (Preblud et al., 1984). The disease is highly contagious and displays a marked seasonality, with most cases occuring during the months of March, April, and May in temperate

climates (Preblud et al., 1984). The pathogenesis of chickenpox is poorly understood, mainly because there is lack of a good animal model that mimics the human disease. The current model of pathogenesis is based on the mousepox model described by Fenner (Fenner, 1948). Briefly, contact with infectious virus occurs through inhalation of infectious respiratory droplets or direct contact with an individual (this may be from an individual with chickenpox or zoster). The virus first replicates locally in the respiratory tract and subsequently disseminates locally to the bloodstream and lymphatics in a primary viremia. The virus is then thought to enter the reticuloendothelial system (RES) where it undergoes multiple rounds of replication, and secondary viremia occurs (Feldman and Epp, 1979). The secondary viremia is followed by infection of capillary endothelial cells with spread to epithelial cells of the epidermis, producing cutaneous and mucosal lesions- a papulovesicular rash. The incubation period before eruption of the papulovesicular rash lasts from 10-21 days, with an average of about 2 weeks. Chickenpox is characterized by many small vesicular lesions that appear throughout the cutaneous areas of the body (mucosal and internal organs may also be involved) with heaviest concentrations around the head and trunk. Lesions appear in distinct waves lasting 2-4 days, whereupon they dry and form a crust that generally heals without scarring. At this time, most normal healthy individuals begin to recover without any other serious

complications. More serious and potentially fatal complications usually are associated with neonates, immunocompromised individuals, and sometimes normal adults (Preblud et al., 1984; Fleisher et al., 1981; Guess, 1986). These complications include bacterial superinfection of the skin, neurologic complications such as varicella encephalitis, and respiratory involvement; eg. varicella pneumonia (Bullowa and Wishik, 1935; Johnson and Milbourne, 1970; Fliesher et al., 1981; Guess, 1986; Weinstein and Meade, 1956). Varicella infection of suseptible pregnant women can result in congenital defects (Paryani and Arvin, 1986; Brunell, 1983). Chicken pox has also been implicated in many cases of Reye's syndrome (Pitel, 1980). Natural VZV infection confers life-long immunity to chickenpox in normal individuals, however, there are a few reported cases of exogenous reinfection resulting in a second episode of chickenpox (Arvin et al., 1983).

The suggestion that varicella and zoster (commonly known as shingles) were both caused by the same etiologic agent was first made by Von Bokay (Von Bokay, 1909). He noticed that household susceptibles contracted chickenpox shortly after exposure to zoster. Susequently, examination of tissues from varicella and zoster skin lesions revealed histologic similarities (Lipschutz, 1921). Discovery of tissue culture methods allowed culture from both varicella and zoster lesions of viruses that had the same growth characteristics and plaque morphology (Weller, 1953). In addition, later

studies using convalescent antisera from both varicella and zoster patients were equally effective in detecting antigens to both varicella and zoster in immunofluorscence, complement fixation and neutralization assays (Weller and Coons, 1954; Weller and Whitton, 1958). Molecular (and final) proof came from restriction enzyme analysis of DNA from primary and reactivation infections from the same individual, where the restriction profiles from both viral isolates were identical (Straus et al., 1984).

After a patient recovers from chickenpox, the virus is not totally eliminated, but is harbored mainly in the sensory nerve ganglia. Both VZV DNA and RNA have been detected in normal human sensory ganglia (Hyman et al., 1983; Gilden et al., 1983). The location of virus in the ganglion is thought to result either from spread of virus from infected skin cells to sensory nerve endings where it ascends to the ganglia, or by hematogenous infection of the ganglion directly during the viremic phase of infection. The state of the virus in the latent phase is unknown, but, recent work by Croen et al. (1988) has shown that it is located in satellite cells of the ganglia, and that limited transcription occurs from selected regions of the genome. Whether this represents virus in a truly latent state, or is a persistent infection is not known. This is in contrast to HSV-1, the genome of which resides in the neuronal cell itself, and transcribes a single RNA molecule (LAT; latency associated transcript) that is antisense to one of its regulatory proteins (Stevens et

al., 1987). For unknown reasons, VZV is able to reactivate from this latent state, and cause the second form of VZV disease, zoster. Zoster is characterized by lesions which are varicellaform in nature but, unlike varicella, are usually unilateral and limited to the innervation of a single sensory nerve. The course of infection differs slightly from varicella. Prodromal symptoms include severe pain, which is then followed by eruption of vesicles over the course of several days. The lesions usually resolve in 2-3 weeks. Complications can result, and the severity of zoster is usually related to the immune condition of the host. Complications include postherpatic neuralgia, myelitis, and encephalitis (Watson and Evans, 1986; Reichman, 1978; Hogan and Krigman, 1973; McCormick et al., 1969).

Zoster can occur at all ages; however, there is a direct correlation between increasing age and increasing incidence of zoster (Hope-Simpson, 1965; McGregor, 1957). This is attributed to a declining cellular immune response rather than lowered humoral immunity, and several studies support this hypothesis. Zoster is known to occur despite demonstrable VZV-specific antibodies (Brunell et al., 1972). A second study found that while antibody levels to VZV membrane antigens remained positive to the ninth and tenth decades of life, delayed hypersensitivity and lymphocyte stimulation responses to VZV antigens began to wane starting at 40 and 60 years of age respectively (Burke et al. 1982). Also at special risk for zoster infections are individuals

who are immunosuppressed from underlying neoplasms like AIDS, Hodgkins disease, or from immunosupressive therapies (Ruckdeschel et al., 1977).

The Infectious Agent

While the clinical descriptions of both varicella and zoster are well documented, knowledge of the biochemical properties and growth cycle of the virus is far more limited. The reason for this is that virus propagation in tissue culture systems currently available for growing VZV in the laboratory are not especially suitable for detailed physical or biochemical studies. Weller et al. (1953) first successfully grew VZV in the laboratory in 1953, propagating the virus in human foreskin fibroblasts (HFF) and in human skin muscle tissue (Weller, 1953). Since then, other cells permissive for growth of VZV have been described, such as human melanoma, human lung embryo fibroblasts and vero cells (Dumas et al., 1980; Grose et al., 1979). However, human diploid cells such as HFF or human embryo lung fibroblasts (MRC-5, WI-38) support VZV growth most readily and remain the cells of choice. As Weller recognized initially, and in contrast to HSV-1 or HSV-2, VZV remains highly cell-associated, and obtaining cell free virus is tedious and difficult. Synchronous infections with cell numbers large enough to carry out classical genetics and biochemistry are not readily attainable. However, enough cell-free virus can be obtained to study the virus particle itself and to obtain

sufficient DNA to be amplified in bacteria and studied directly.

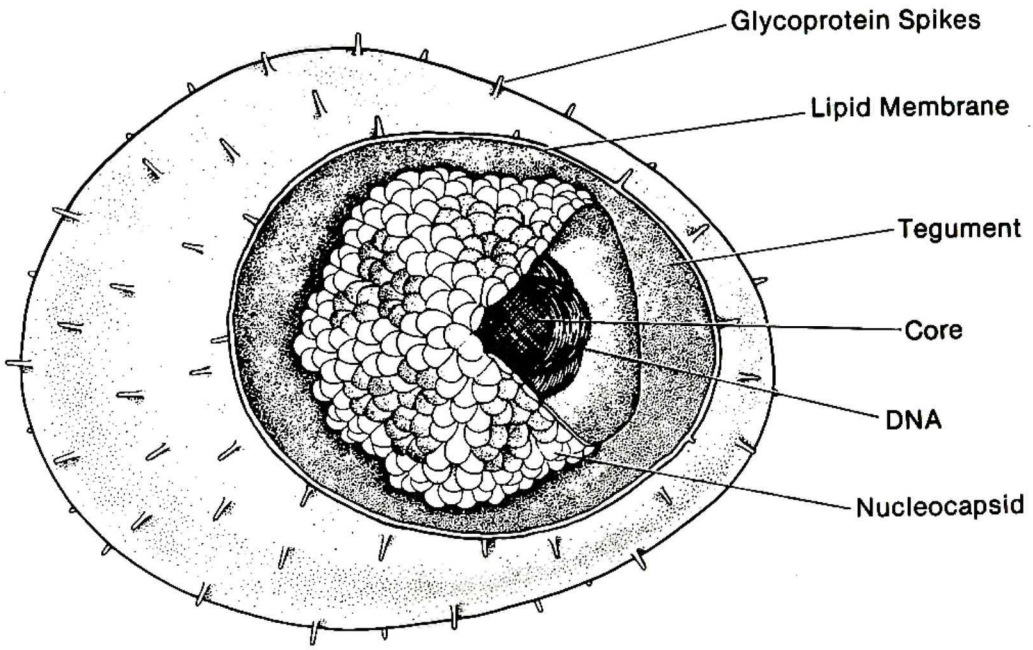
VZV resembles other members of the herpesvirus family in morphology (Almeida et al., 1962). The virus particle is 0.125-0.175 μm in diameter, and consists of several structures. The outermost layer is an irregular membrane containing glycoprotein projections. Enclosed within the membrane is an icosehedral nucleocapsid consisting of 162 capsomeres. Between the nucleocapsid and the outermost membrane is a granular proteinaceous material called the tegument. The DNA genome is organized in a toriodal shaped protein core within the nucleocapsid. These structures are shown in Figure 1.

While the morphology of VZV is similar to that of the other human herpes viruses, it is really the genome and its genetic potential that gives VZV its specific properties, distinguishing it from other herpesviruses.

VZV DNA from many clinical isolates has been purified using several techniques and analyzed by restriction endonuclease profiles, electron microscopy, and buoyant density (Straus et al., 1981; 1982; 1983; Dumas et al., 1980; Davison and Scott, 1983; Ludwig et al., 1972; reviewed in Gelb, 1990). All of these studies culminated in 1986 with the publication of the entire sequence of the VZV genome (Davison and Scott, 1986b). In summary, these data indicate that the VZV genome is comprised of a linear double-stranded DNA molecule approximately 125,000bp in length. The G+C

FIGURE 1

Structural model of the herpesvirus virion. A cutaway diagram of a virion showing the individual structural components. (reproduced from Nermut and Steven, 1987)

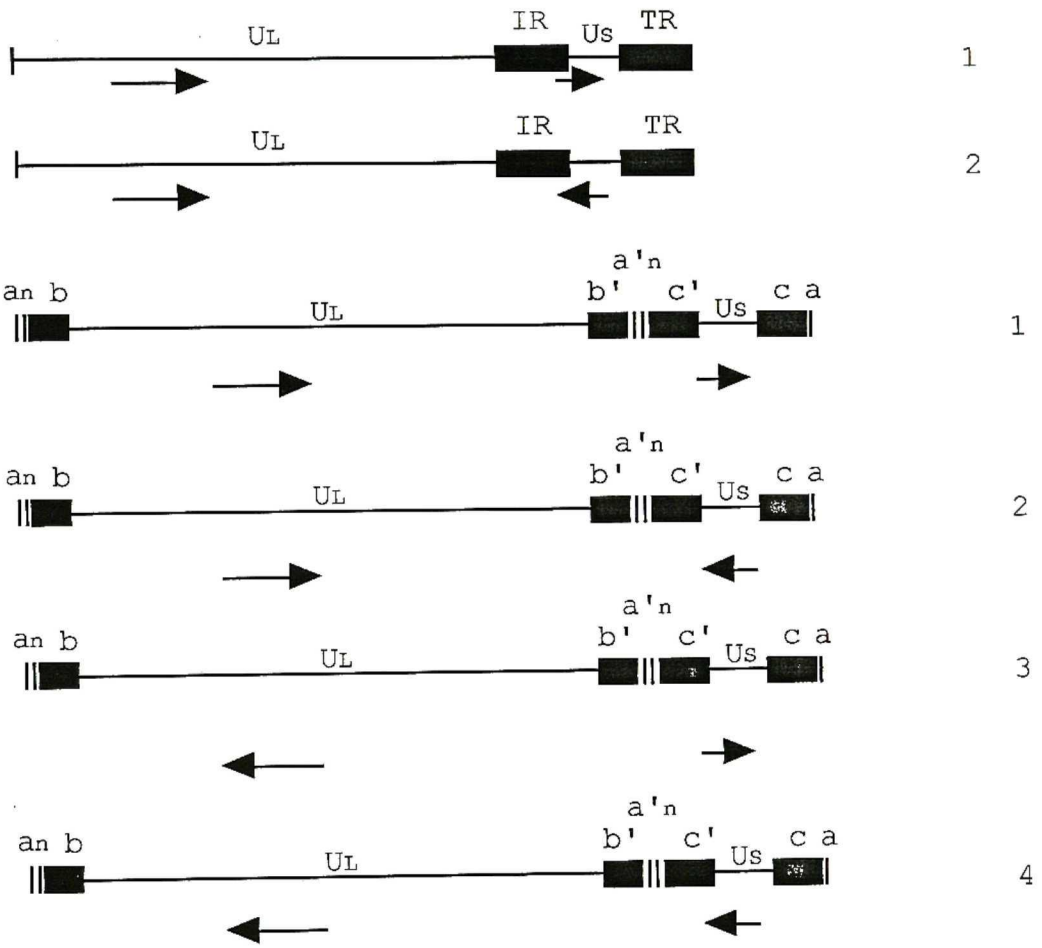

Glycoprotein Spikes
Lipid Membrane
Tegument
Core
DNA
Nucleocapsid

content is about 47%. The genome itself consists of two unique segments, one long (designated U_L) and one short (U_S). Two short repeated segments of 80-90bp are found at the termini of U_L and much longer reiterated sequences are found on the U_S termini (7319bp) designated IRs (internal repeat short) and TRs (terminal repeat short), both of which are identical (see fig. 2). The U_S segment can invert (approximately 50% of the time) resulting in two different isomeric forms of the genome. While inversion can occur for the U_L segment, it occurs much less frequently (about 2-5% of the time) (Kinchington et al., 1985). Thus, two isomers exist predominantly for the genome structure (see fig. 2). HSV has a genome structure similar to that of VZV except that the terminal sequences for its U_L segment are considerably larger (McGeoch et al., 1988). In addition, the HSV genome has an equal frequency of U_S and U_L inversions resulting in equimolar ratios of four isomeric forms of the genome (see fig. 2) (Reviewed in Roizman and Sears, 1990).

The published sequence analysis of the VZV genome reveals 71 putative open reading frames (ORFs), of which 68 are unique (some ORF are present twice in the repeated segments) (Davison and Scott, 1986b). These ORFs have been numbered, starting with 1 at the left hand end of the U_L segment sequentially to the right hand end of the TRs (Davison and Scott, 1986b). A few of these ORFs had been mapped prior to the sequence analysis, but the characteristics of most of the potential ORFs remain unknown (Sawyer et al., 1986; Felser

FIGURE 2

Diagram of VZV and HSV genome structures. The top two figures represent two isomeric forms of the VZV genome, and the bottom four figures show the four isomeric forms of the HSV genome.


UL
IR
Us
TR
1
UL
IR
TR
2
an b
a'n
b'
c'
c a
UL
Us
1
an b
a'n
b'
c'
c a
UL
Us
2
an b
a'n
b'
c'
c a
UL
Us
3
an b
a'n
b'
c'
c a
UL
Us
4

et al., 1987; Keller et al., 1986; Ellis et al., 1985). Comparison of the primary amino acid sequences of ORFs from VZV with those of HSV (the HSV-1 genome has also been sequenced in its entirety) reveals many similarities (Davison and Scott, 1986b; McGeoch et al., 1988). Interestingly, many of the VZV proteins share homology to proteins in HSV, and their locations in the genome are also very similar. In this respect, given the similarity in genome structure and location of homologous genes, both viruses are considered to be grossly co-linear (reviewed in McGeoch, 1989). One confusing aspect of comparisons made between these two viruses is that the arbitrary designation of prototype arrangements has the U_L segments in opposite orientations. Thus, the prototype arrangements for VZV and HSV are isomer #1 for VZV and isomer #3 for HSV as shown in figure 2.

Identification of VZV encoded proteins

While analysis of the viral DNA and the virus particle can be approached directly, the regulated processes of the virus life cycle and identification of polypeptide function are much more difficult to study without good tissue culture or genetic (e.g. mutant) systems. Nevertheless, several approaches have been successful in identifying and mapping VZV encoded gene products. In one instance, the viral thymidine kinase gene was mapped by transfecting cloned viral DNA fragments into cells lacking thymidine kinase activity and selecting for cells able to grow in medium containing

hypoxanthine, aminopterin, and thymidine (Sawyer et al., 1986). DNA fragments (whose map locations were known) able to confer cell survival contained the thymidine kinase gene. In another instance, investigators had prepared monoclonal antibodies that recognized an antigenically distinct group of glycoproteins, designated gpII, found in VZV-infected cells (Keller et al., 1984). RNA extracted from VZV-infected cells was hybrid-selected using cloned DNA fragments from the genome; the hybrid-selected RNA was then translated *in vitro*. The *in vitro* translation products were immunoprecipitated with the gpII monoclonal antibodies. Only the fractions of hybrid-selected RNA complementary to the Hind III D DNA fragment were able to code for polypeptides reactive with the gpII monoclonal antibody, thus mapping gpII near the center of U_L (Keller et al., 1986).

Subsequent knowledge of the VZV sequence has made identifying and mapping VZV genes and their polypeptide products much easier. Davison et al. (1985) were able to identify products of another putative glycoprotein gene, gpIV, by synthesizing oligopeptides derived from knowledge of the nucleotide sequence of the putative gpIV ORF. The oligopeptide was injected into rabbits and the resulting antisera immunoprecipitated specifically 45K and 55K glycoproteins made in VZV-infected cells but not uninfected cells. We have used a different strategy to identify products of a fifth glycoprotein (gpV) predicted from sequence analysis, and this is described in this

dissertation. A final example is identification of the VZV thymidylate synthetase gene (TS). VZV nucleotide sequence analysis revealed an ORF whose primary amino acid sequence was homologous to other previously identified TS genes (Davison and Scott, 1986b). This ORF was cloned into a prokaryotic expression vector, which was then able to complement *E. coli* from which the natural TS gene had been deleted (Thompson et al., 1987). In addition, an isotopically-labeled ligand binding specifically to TS polypeptides was able to detect in VZV-infected cells a TS that was the same apparent molecular weight as the product made from the prokaryotic expression vector but different from host cell TS (Thompson et al., 1987). Several other proteins such as a VZV-encoded ribonucleotide reductase, DNA polymerase, major DNA binding protein, and major capsid protein have been identified based on amino acid homology to known proteins found in other herpesviruses (Davison and Scott, 1986b; Kinchington et al.,1988).

VZV gene expression

Herpesviruses, in general, express gene products in a sequential manner during their life cycle in an infected cell (Roizman and Sears, 1990). This temporal order of expression has been divided into 3 groups (Honess and Roizman, 1974). The first class of genes expressed in infection is the immediate early (IE) or α genes. The second is the early (E) or β genes, which is then followed by synthesis of late (L)

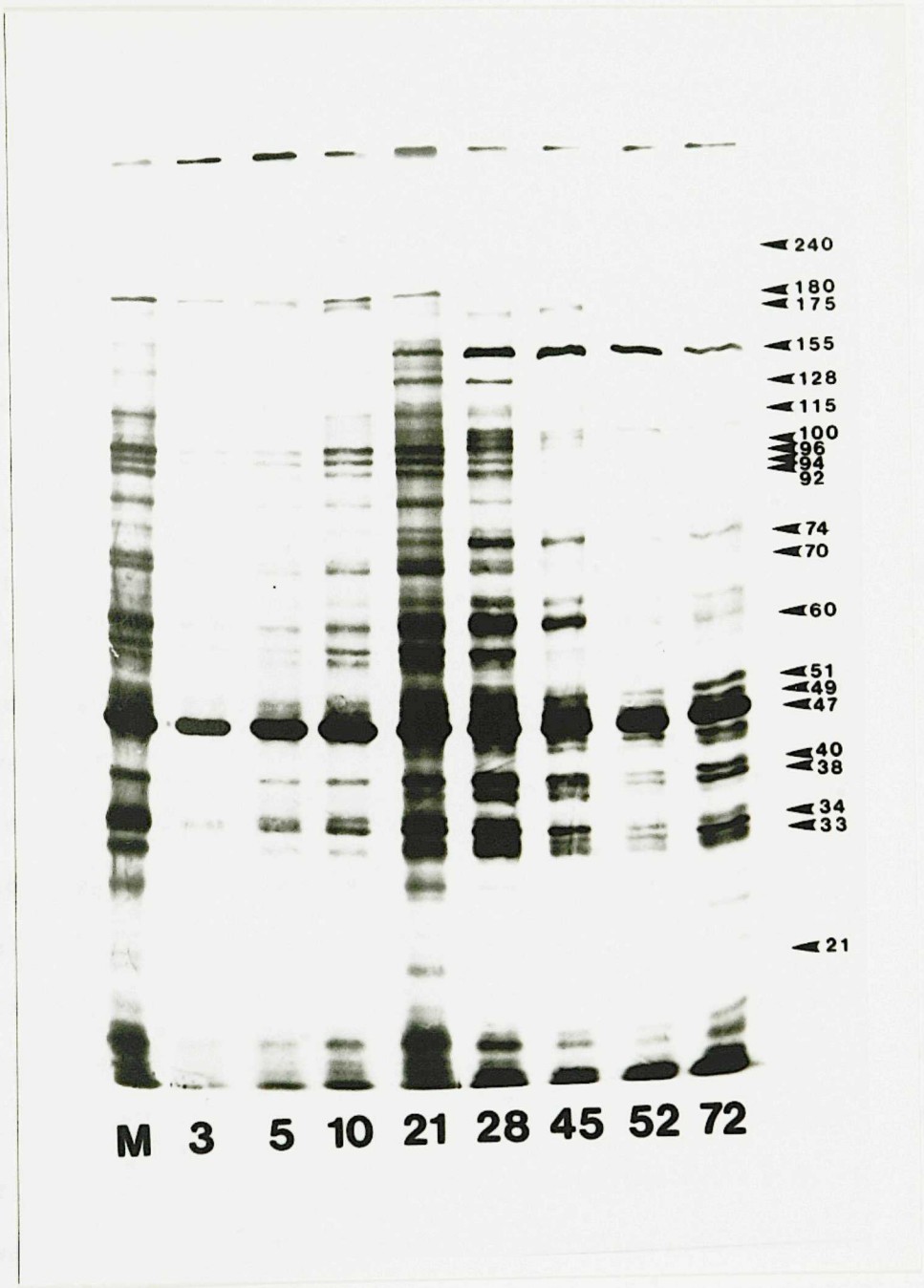
or γ genes. To avoid confusion, the "immediate early", "early", "late", nomenclature will be used for discussion of the viral gene classes. The classes of gene expression are defined on the basis of whether or not a gene is transcribed in the presence or absence of certain metabolic inhibitors (Honess and Roizman, 1974). The immediate early (IE) genes are defined by their ability to be transcribed in the absence of cellular (and also viral) protein synthesis, e.g., in the presence of cycloheximide. Removal of cycloheximide will allow translation of IE genes and transcription of the second temporal class of genes (early, E) class, can begin shortly thereafter. These genes are not expressed in the absence of competent IE gene products. Finally, the third temporal class, the late (L) genes, require DNA synthesis for their expression in that they are not made in the presence of viral DNA synthesis inhibitors such as phosphonoacetic acid (PAA). It is generally accepted that the coordinate regulation of herpesvirus gene expression is at the level of transcription initiation; it should be noted, however, that one exception has been documented in cytomegalovirus (CMV) (Geballe and Mocarski, 1986).

Classic kinetic labeling experiments at high multiplicities of infection were performed in determining the established pattern of protein synthesis in HSV (Honess and Roizman, 1974). However, identification of temporally regulated polypeptide synthesis has yet to be fully characterized in VZV. The major difficulty has been, as

discussed previously, to obtain enough cell free VZV to make this type of study feasible. In order to obtain extensive, synchronous infections, with VZV, Ruyechan et al. (1990) and others (Asano and Takahashi, 1979) have infected cell monolayers with high ratios of infected cells. Experiments from our laboratory used a ratio of 1 infected to 4 uninfected cells; cells were labeled with ^{35}S-methionine and harvested at various intervals over a 72 hr. time period. The cell extracts were then analyzed by SDS-PAGE and autoradiography. In VZV-infected cells four novel polypeptides appeared at 10 hrs. post-infection ranging from 21 kDa to 175 kDa in size and are probable IE proteins. At 21 hrs. post-infection, a second set of four novel polypeptides appeared ranging from 35 kDa to 128 kDa in size and are candidate E proteins. In both cases, these novel polypeptides disappear late in infection. Finally, a third set of approximately 10 polypeptides appeared, ranging in size from 33-175 kDa between 21 and 28 hrs. post-infection and continue to be produced throughout the remainder of the infection. These results can be seen in figure 3. Work by Asano and Takahashi (1979) agrees with these results, although the number of observed polypeptides and their appearance in infection are slightly different from our data. This is probably due to variables, such as inability to control the initial infection inoculum accurately. In any case, these results indicate that VZV does in fact encode polypeptides whose expression can be divided into three

FIGURE 3

Time course of VZV polypeptide synthesis. Autoradiogram of ^{35}S-methionine-labeled polypeptides synthesized in VZV-infected cells over a 72 hour time course. Lane M: extract of mock-infected human foreskin fibroblast cells (HFF) labeled 10-21 hours post mock infection; Lane 3: polypeptides produced 0-3 hours post infection; Lane 5: polypeptides produced 3-5 hours post infection; Lane 10: polypeptides produced 5-10 hours post infection; Lane 21: polypeptides produced 10-21 hours post infection; Lane 28: polypeptides produced 21-28 hours post infection; Lane 45: polypeptides produced 28-45 hours post infection; Lane 52: polypeptides produced 45-52 hours post infection; Lane 72: polypeptides produced 52-72 hours post infection.
(reproduced from Ruyechan et al., 1990)


240
180
175
155
128
115
100
96
94
92
74
70
60
51
49
47
40
38
34
33
21
M
3
5
10
21
28
45
52
72

temporal classes. In addition, Asano and Takahashi (1979) carried out kinetic labeling experiments similar to those described above which included the DNA synthesis inhibitor PAA. A number of specific novel polypeptides observed in infected cells were not observed in PAA-treated infected cells. Shiraki and Hyman (1987) infected cells with cell-free VZV and conducted cycloheximide "block and release" followed by addition of actinomycin D (to block RNA synthesis of E gene products) and observed four putative IE proteins with molecular weights of 185, 69, 43, and 34 kDa. The larger 3 species were also identified as phosphoproteins. These data also confirm that VZV gene products can be conventionally classified as in other herpesvirus infections, based on their ability to be produced or not under well-defined metabolic conditions.

Nevertheless, with the exception of the Shiraki study, these experiments have severe problems. Infection with infected cell material can bring in a background of cells actively synthesizing viral proteins that may confuse results. In addition, cellular protein synthesis in infected cells is not inhibited to the extent found in HSV infected-cells, giving a high background of host cell proteins. Until better methods are available for obtaining cell-free virus, or assaying for kinetic classes, assignment of VZV proteins to kinetic classes is probably best drawn from parallels with homologous proteins found in HSV.

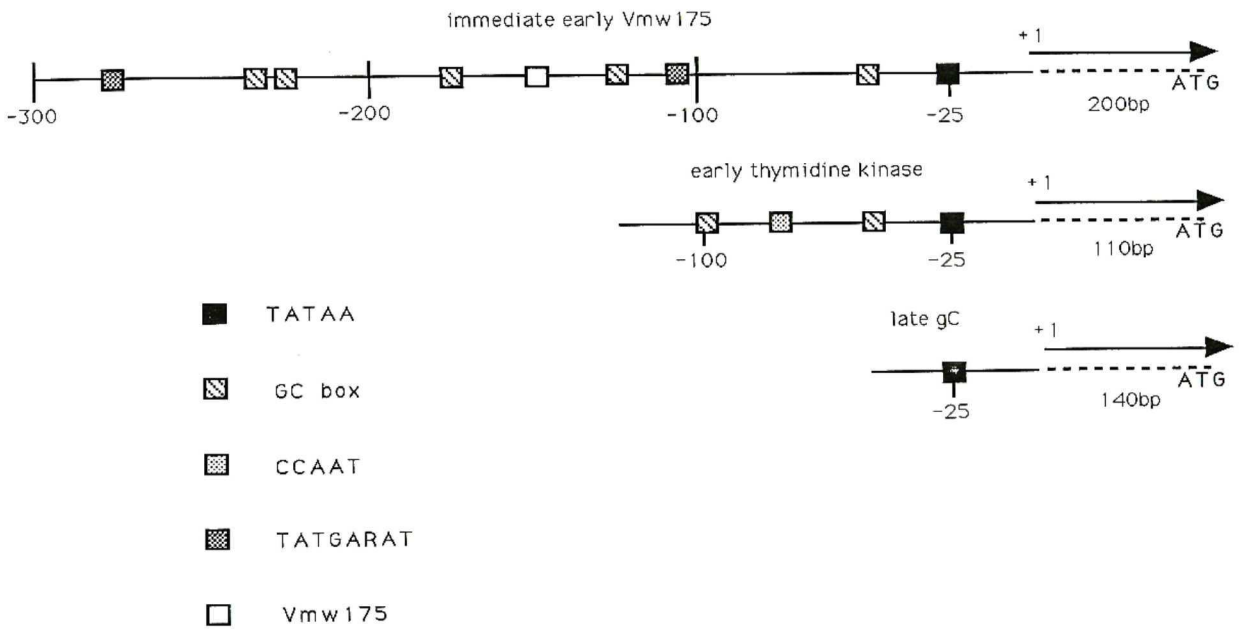
Studies of coordinate regulation of herpesvirus gene expression have been the focus of intense investigation. Primarily, these have involved the localization of transcripts and their termini, as well as RNA spicing. Generally, in contrast to host cell transcription, HSV RNAs are not spliced, but are capped, polyadenylated, and are synthesized by host cell RNA polymerase II (Bachenheimer and Roizman, 1972; Bartkoski and Roizman, 1976; Costanzo et al., 1977). Transcript data have been used to localize likely promoter regions for all kinetic classes of genes, while the analysis of HSV promoters has centered on defining the minimum promoter domains, and cis-acting sites that confer authentic viral regulation on the target gene. In their simplest forms, HSV promoters contain sequence elements similar to host cell promoters, along with somewhat less well-characterized sequence elements that appear to be viral specific. Immediate early gene promoters, such as the one for Vmw175 (virus protein molecular weight 175 kDa) contain a TATAA box, several GC-rich (CCCGCC) binding sites, a TATGARAT motif (where R is any purine), and sequences that bind the Vmw175 gene product itself (ATCGTCnnnnYCGRC) (Kristie and Roizman, 1984; 1986; Mackem and Roizman, 1982a; Mackem and Roizman 1982b; Jones and Tijan 1985a and b). Detailed analysis of the thymidine kinase (an early protein) promoter, has shown that it contains a CCAAT box, two GC-rich binding sites, and possibly an octamer motif (ATTGCAT) (Mcknight et al., 1981; 1982a and b; 1984). In addition, the Vmw175 gene

product can also bind to this promoter, but the binding sites are not well characterized (Roberts et al., 1988; Deluca and Schaffer, 1988). Finally, analyses of the gC (a late protein) promoter, has revealed that only the TATAA box and perhaps some 5' untranslated regions are present (Homa et al., 1986a, 1988). The structure of these promoters is shown in figure 4. Efforts to categorize promoters based soley on sequence examination are difficult, however, since promoters from many of the kinetic classes contain a high degree of heterogeneity. For example, only IE promoters for the Vmw175 and Vmw110 genes contain the TATGARAT sequence. The examples in Figure 4 depict the most rigorously studied promoters, and are shown primarily for comparative purposes.

The interaction of host cell and viral encoded proteins with these promoters is complex, and their role in assisting RNA polymerase loading and subsequent transcription is still unclear. However, host cell proteins binding to the TATAA (TFIID), GC-rich motifs (SP1), CCAAT (CTF/NF1, C/EBP), and TATGARAT (αH1/OTF-1/NFIII) sequences have been identified (Schmidt et al., 1989; Horikoshi et al., 1989; Santoro et al., 1988; Sturm et al., 1988; Kadonaga et al., 1987; reviewed in Mitchell et al. 1989). Vmw175, a potent transcriptional transactivator, has been demonstrated to bind to its own promoter as well as to early and late promoters, however, the binding sites in these promoters are not well characterized (Michael et al., 1988). Another viral protein, Vmw65, interacts with host cell factors (see above) through

FIGURE 4

Diagram of three HSV promoters. The diagram shows three HSV promoter regions. The start of transcription is shown as +1 and the leader distance is shown below in base pairs. Various sequence elements identified as being important are shown as shaded boxes. The distance in base pairs from the cap site is shown in negative numbers. The key for each sequence element is shown to the bottom left of the figure.


immediate early Vmw175
+1
ATG
200bp
-300
-200
-100
-25
early thymidine kinase
+1
ATG
110bp
-100
-25
late gC
+1
ATG
140bp
-25
TATAA
GC box
CCAAT
TATGARAT
Vmw175

the TATGARAT motif to dramatically enhance transcription, but does not bind DNA directly itself (McKnight et al., 1987; Preston et al., 1988; Triezenberg et al., 1988) Finally, several other viral IE proteins, Vmw110, Vmw68, and Vmw63 and one early gene (the major DNA binding protein) also appear to regulate transcription of viral genes, but the detailed mechanisms behind their action are still unknown (Stow and Stow, 1986; Sacks et al., 1985; Sacks and Schaffer, 1987; O'Hare and Hayward, 1985; McCarthy et al., 1989; Everett, 1986; Godowski and Knipe, 1983; Post and Roizman, 1981; reviewed in Roizman and Sears, 1990).

While knowledge of VZV lags behind HSV, several recent have begun to characterize VZV transcription and gene regulation. Analysis of RNA from VZV-infected cell extracts has led to identification of more than 70 abundant transcripts ranging from 0.8kb to 8.0kb in size (Ostrove et al., 1985; Reinhold et al., 1988; Maguire and Hyman, 1986). The majority of these transcripts are polyadenylated although two species were not; the function of these nonpolyadenylated transcripts is unknown (Maguire and Hyman, 1986). The directionality and size of these transcripts correlate roughly with predicted ORFs based on VZV sequence analysis. Two ORFs (42 and 45) are likely to be spliced based on the presence of consensus donor and acceptor sites, however, these transcripts have yet to be identified (Davison and Scott, 1986b). In contrast to these studies, there is little information available on the detailed structure of VZV

transcripts. Only two reports of genes that have been transcriptionally mapped in some detail have been published. The first VZV transcript to be studied in some detail was that derived from the VZV pyrimidine deoxynucleoside kinase (dPyK) gene. This 1.8kb transcript was first identified by Sawyer et al.(1986) and subsequently, Davison mapped the 5' and 3' ends of this transcript (Davison and Scott, 1986b). Several interesting features emerge from these studies. A putative 'TATA' box (TATTAA) occurs 25bp upstream of the RNA initiation site, however, three additional A-T rich concensus 'TATA' elements reside between the 5' end of the transcript and the AUG initiation codon. Thus, the true 5' RNA terminus could not have been predicted from examination of the sequence alone. Similarly, the 3' terminus resides just downstream from an AGTAAA sequence, which is 119bp and 266bp upstream from other canonical polyadenylation sequences (AATAAA and ATTAAA). The 5' untranslated region is 420bp long, compared to a 110bp leader in the equivalent HSV gene. In addition, 3 AUG sequences in different open reading frames are present in the leader sequence, each of which is followed by a translational termination signal. The relevance of these small ORFs (if any) is unknown. A second gene transcript, that for ORF62, has also been mapped. It was first identified as a 4.3kb transcript by Felser et al. (1988), and S1 nuclease and primer extension mapping has localized its 5' termini (McKee et al., 1990). A 'TATA' element (TTTTAA) resides 25bp upstream from the transcript

initiation site. As in the VZV dPyK gene, several other 'TATA' consensus sequences are located further upstream and were predicted to be the likely elements used before the more detailed transcript mapping results became available. Results from the dPyK gene have caused Davison and his colleagues to suggest that transcriptional (and perhaps translational) control of homologous VZV and HSV genes may be considerably different (Davison et al., 1986b). Several reports have now been published that support their hypothesis, at least in part. For example, McKee et al. (1990) examined sequences found upstream from the VZV ORF62 transcript initiation site and found, as in its HSV homologue Vmw175, a TATGARAT-like sequence. In transient assays, VZV ORF62 promoter constructs containing this motif were transactivated by the HSV Vmw65 gene. However, when the Vmw65 homologue in VZV (ORF10) was used, either to stimulate the HSV Vmw175 promoter or the VZV ORF62 promoter, no activation was observed. One additional interesting finding was that the activity of the ORF62 promoter in transient expression assays under all circumstances was at least 2 orders of magnitude less than its HSV counterpart. Thus, the sequence motifs found upstream from the VZV ORF62 transcription initiation site exist for potential transactivation by Vmw65, but, the VZV homologue to Vmw65, ORF10, lacks transactivation functions.

Several elegant studies by Felser et al. (1987, 1988) examined the functional activity of the ORF62 product. These

studies demonstrated that VZV was able to complement HSV *ts* mutants carrying a lesion in the Vmw175 gene. Felser then went on to map the complementation activity to the VZV ORF62 gene product, which from amino acid homology and genome location, was predicted to be the Vmw175 homologue. In subsequent studies from the same laboratory, Inchauspe et al. (1989a) were able to demonstrate that the VZV ORF62 gene product could stimulate transcription from the VZV dPyK and glycoprotein I (gpI) promoters in transient assays (Inchauspe et al. 1989a). These studies also demonstrated that the VZV ORF4 gene product together with the ORF62 gene product could stimulate transcription from these promoters in a synergistic fashion. These results are reminiscent of studies using the homologous genes in HSV (Vmw175 and Vmw63) that showed transactivation of a variety of HSV promoters (Everett, 1986; O'Hare and Hayward, 1984; Shapira et al. 1987). Unlike its homologue in HSV, the VZV ORF61 gene product has been demonstrated to have no transactivating potential, and perhaps a negative regulatory activity (Inchauspe et al., 1989a; Cabriac et al., 1990). The HSV ORF61 homologue, Vmw110, is in contrast a powerful general transactivator of transcription (O'Hare and Hayward, 1984).

In summary, it appears that some VZV proteins, for example ORF62 and ORF4, have similar functional activities to those of their HSV homologues, while others such as ORF10 and ORF61 do not. In addition, the detailed transcript analyses of two VZV genes also suggested that predicted sequence motifs that

may be involved in transcription found in the VZV genome do not necessarily correlate to transcription mapping results or to previously defined eukaryotic sequence elements.

The live attenuated VZV vaccine virus

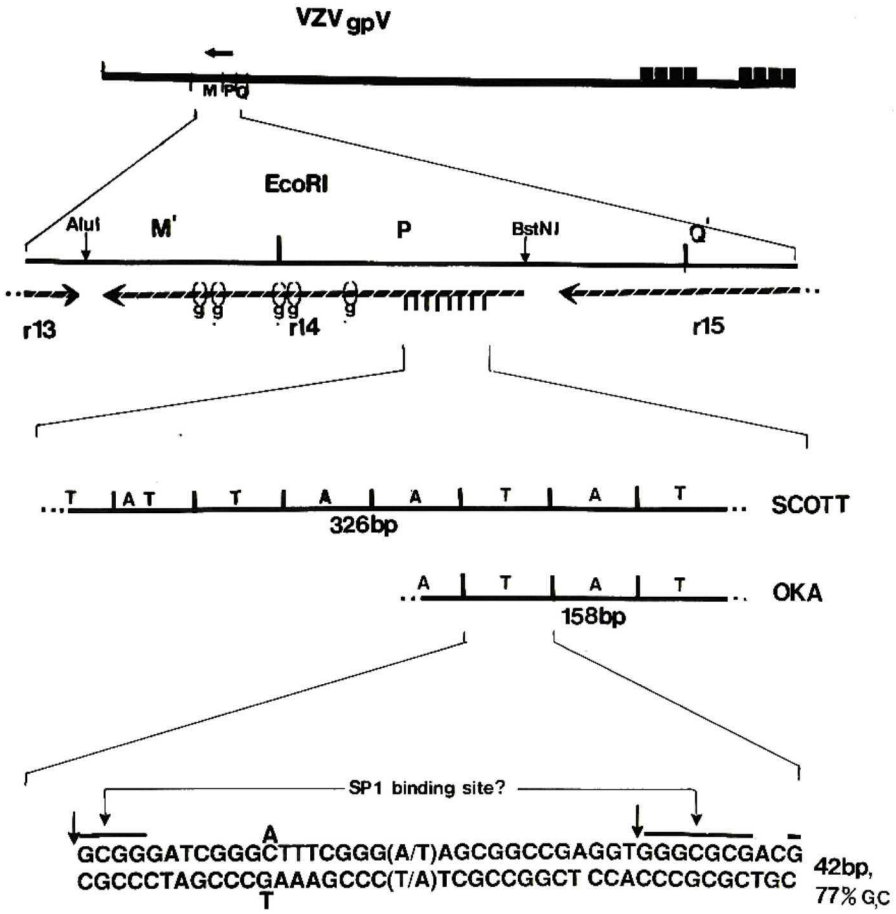
The live attenuated VZV vaccine virus (LAVV) was originally developed in 1974 by Takahashi and co-workers (1975). The vaccine was developed from virus isolated from a young boy with chickenpox, whose name was Oka. This Oka virus was passaged 11 times at 34^0C in human embryonic lung cells and then passaged 12 times at 37^0C in guinea pig embryo cells. The virus was then passaged several more times in human diploid cells (WI-38 and MRC-5), whereupon cell free virus was prepared for vaccine trials. The original target for the vaccine was children who had immune impairments due to leukemia. Chickenpox can be a severe, life-threatening disease for these individuals and in a hospital ward setting can spread rapidly among these children (Cheatham et al., 1956). Clinical trials with the Oka vaccine have been successful in immunizing children with leukemia. Recently, it has been proposed that the vaccine be routinely used to immunize the general pediatric population and be combined with the MMR (measles, mumps, rubella) vaccine (Arbeter et al., 1986).

While there is considerable data available on the use of LAVV as a general vaccine, we know very little about the biochemical mechanisms that make it attenuated. Takahashi

(1984) has shown that compared to wild-type strains the vaccine strain is slightly temperature sensitive at 39^{0}C relative to 34^{0}C. In addition, these investigators found a restriction length polymorphism (RFLP) compared to the Oka parental strain upon digestion with Hpa I, mapping to the Hpa I K fragment. Ecker and Hyman (1981) also showed RFLP between the parent Oka and LAVV. Previous work in our laboratory also found an RFLP mapping to the EcoR I P fragment, that varied in size between a lab strain Scott, and Oka (LAVV) (Kinchington et al., 1986). This size variation was further characterized by sequence analysis. The results showed that the EcoR I P fragment contained a portion of an open reading frame that had the characterisics of a glycoprotein. This turned out to be a previously unidentified VZV glycoprotein that had some amino acid homology at its COOH-terminus to glycoprotein C of HSV. Under the nomenclature for VZV glycoproteins this has now been named glycoprotein V (gpV). The gpV ORF contains a number of 42bp repeating elements (R2) responsible for the observed RFLP in the EcoR I P fragment. Strain Scott has 7.5 copies of R2, whereas Oka has 3.5 copies, resulting in a 168bp deletion in the Oka gpV ORF. The results of these studies are summarized in Figure 5. This difference between Scott and Oka has been further characterized, and is part of the subject of this dissertation.

FIGURE 5

Diagram of the gpV open reading frame with different copy numbers of intragenic 42bp repeats in Scott and Oka. The top of the figure shows the VZV genome with an arrow designating the location and direction of the gpV ORF. The EcoR I P restriction fragment is expanded below showing the gpV ORF; the vertical lines near the BstN I restriction site are the 42bp repeats. These 42bp repeats are expanded below showing the size difference between the Scott and Oka strains in this portion of the sequence. The sequence of a 42bp repeat is shown at the bottom of the figure. (reproduced from Kinchington et al., 1986)


VZV gpV
M
P
Q
EcoRI
AluI
M'
P
BstNI
Q'
r13
r14
r15
T A T T A A T A T
326bp
SCOTT
A T A T
158bp
OKA
SP1 binding site?
GCGGGATCGGGCTTTCGGG(A/T)AGCGGCCGAGGTGGGCGCGACG
CGCCCTAGCCCGAAAGCCC(T/A)TCGCCGGCT CCACCCGCGCTGC
42bp,
77% G,C

Specific Aims

When the present work began (1986), 4 VZV glycoproteins had been identified and mapped (Ellis et al., 1985; Keller et al., 1986, Keller et al., 1987; Davison et al., 1985). Sequence analyses in our laboratory subsequently suggested that a fifth glycoprotein, gpV, also was encoded in VZV (Kinchington et al., 1986). Knowledge of glycoprotein structure and function is particularly important in understanding viral pathogenesis, the growth cycle and the host immune response. Knowledge of glycoprotein expression also is important in understanding the regulatory events controlling viral gene expression.

The aims of this work were three-fold. First, to identify the transcripts encoding glycoproteins gpIV and gpV, and test potential regulatory sequences controlling transcription of these genes; second, to identify polypeptide products from the gpV gene and confirm the glycoprotein nature of these products and finally, to test for viral functions that may be associated with these glycoproteins, based on knowledge of their homologous counterparts in HSV.

MATERIALS AND METHODS

Cells and Viruses

Human Foreskin Fibroblasts (HFF; strain USU 521) and VZV strain Scott were obtained from Monroe Vincent, Department of Pediatrics, USUHS. MRC-5 cells (ATCC CCL 171), WI-38 cells (ATCC CCL 75), Vero cells (ATCC CCL81), CV-1 cells (ATCC CCL 70), and VZV strain Oka (ATCC VR-795) were purchased from the American Type Culture Collection, Rockville, Maryland. The parent Oka strain was obtained from Michiaki Takahashi, Department of Virology, Research Institute for Microbial Diseases, Osaka University, Yamada-Kami, Suita Osaka, Japan. Three VZV Oka strains used currently as vaccines were obtained from Dr. Lawrence Gelb, Division of Infectious Diseases, Washington University School of Medicine, St. Louis, Missouri.

HFF cells were grown in 150cm^2 flasks (Costar) or 850cm^2 glass roller bottles (Bellco) in Minimum Essential Medium (Gibco) supplemented with 2% (v/v) fetal bovine serum (Gibco), 8% (v/v) Serum Plus (Hazelton Research Products), and 50 µg/ml Gentamycin at 37^{0}C and 5% (v/v) CO_2. When cell monolayers reached confluency they were trypsinized (0.25% in buffered saline, Hazelton), resuspended in growth medium, and seeded into new flasks or roller bottles. All other cell lines were grown in essentially the same manner.

VZV virus was grown by trypsinizing infected cell cultures and adding them to uninfected cell monolayers at a ratio of 1

to 5 infected to 10 noninfected cells. HFF cells were the primary cell line used to propagate VZV, however MRC-5 and WI-38 cells are also permissive for VZV and were used for special applications such as transfection assays. Infected cells were then incubated at 37^{0}C for 3 to 7 days until the cytopathic effect (C.P.E.) as observed under a light microscope reached approximately 80%. Cells were then harvested for experimental use or used to infect other monolayers.

Preparation of VZV nucleocapsids

VZV nucleocapsids were prepared as reported by Straus et al. (1981). Briefly, VZV infected cells were harvested and centrifuged at 1500g for 10 minutes at 4^{0}C. The supernatant was discarded and the cell pellet was frozen and thawed 3 times in a 37^{0}C water bath and a dry-ice ethanol bath. An equal volume of 2x lysis buffer consisting of 1% (v/v) Nondiet P-40, 7.2 mM calcium chloride, 10 mM magnesium acetate, 250 mM potassium chloride, 1 mM EDTA, 12 mM β-mercaptoethanal, and 1% (w/v) sodium deoxycholate was added to the cell pellet. To this solution 25 µg/ml of DNase (Sigma, St. Loius, MO) and 25 µg/ml of RNase (Sigma, St. Louis, MO) were added and the lysate was incubated at 37^{0}C for 30 minutes. The lysate was then extracted with 0.5 ml of Genesolv D (trichloro-trifluoroethane; Aldrich HPLC grade) and centrifuged at 1500g for 10 minutes, at 4^{0}C. The aqueous layer was then loaded on top of a glycerol step gradient (5%

(v/v) and 40% (v/v) glycerol in 2x lysis buffer) and centrifuged in a SW 41 rotor at 40,000 r.p.m. for 40 min. at 4^0C. The supernatant was aspirated and the pellet was taken up in TE, pH 7.2 (TE; 10 mM Tris-HCl, pH 7.2, 1 mM EDTA).

Purification of Viral DNA

Virus nucleocapsids that had been resuspended in TE were then made up to 2% (w/v) SDS, 1 mg/ml of proteinase K, and incubated at 50^0C for 1 hr. Phenol was added to this suspension at an equal volume, mixed gently to minimize shearing forces, and spun in a microfuge to facilitate separation of the aqueous layer. This same process was repeated on the aqueous layer with equal volumes of phenol/chloroform and chloroform. To the final remaining aqueous solution was added 1/10 volume sodium acetate pH 4.8, and 1 µl of glycogen (20 mg/ml; Boehringer Mannheim, Indianapolis, IN). DNA was then precipitated with addition of 3 volumes of cold (-20^0C) ethanol. The precipitated DNA was recovered by centrifugation at 12,000g in a Beckman microfuge for 20 min. at 4^0C . The ethanol was aspirated, the pellet was dried under vacuum, and resuspended in TE, pH 7.2. The amount of DNA was then quantitated by measuring the absorbance at 260nm.

Isolation of RNA

RNA was isolated from uninfected or VZV infected HFF cells by an adaptation of a method by Chirgwin *et al*. (1979) and

Glisin et al. (1974) (Ostrove et al., 1986). Cells were first washed 3 times with ice cold PBS (phosphate buffered saline pH 7.0) and after the final wash all excess PBS was aspirated completely. The cell monolayer was then lysed with RNA lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.1 M ß-mercaptoethanol, and 0.44% [w/v] sarkosyl). The lysate was then layered onto a 5.7 M CsCl cushion and centrifuged in a SW41 rotor at 33,000 r.p.m. for 24 to 30 hrs. The tubes were aspirated of all liquid and the resulting pellet was resuspended in the RNA lysis solution without sarkosyl, brought to a final of 0.2 M in potassium acetate pH 5.0 and precipitated with 2.5 volumes of absolute ethanol. The RNA was spun in a microfuge at 12,000g for 20 minutes, 4^0C. The ethanol was aspirated and the RNA resuspended in water. The RNA was then deproteinized by extraction with phenol and then with phenol/chloroform. The final aqueous layer was then re-ethanol precipitated, spun, and resuspended in water. RNA was quantitated by reading its absorbance at 260nm.

Preparation of protein lysates

HFF cells that were uninfected or infected with VZV were washed directly in the flask with ice cold 1x PBS 3 times. The PBS was then drained and the cells were disrupted with 1x lysis buffer (0.23% (w/v) SDS, 1% (v/v) glycerol, 1.4 M ß-mercaptoethanol, and 0.1 mg/ml bromphenol blue). The lysate was then used directly for SDS-PAGE analysis.

Gel Electrophoresis

Agarose gel electrophoresis: Agarose slab gels were prepared by calculating the desired percentage of agarose (w/v) in 300 ml, and adding the agarose powder to 270 ml of distilled water and 30 ml of 10x TBE running buffer (TBE; 0.089 M Tris, 0.089 M boric acid, 2 mM EDTA, ph 8.0). The agarose was dissolved by boiling in a microwave oven. The solution was cooled to 60^0C, poured in a plastic 20 x 25 cm casting tray (BRL, Gaithersburg, Md.) and then the desired comb inserted. When the gel hardened, the comb was removed and the gel was submerged in an electrophoresis rig (BRL, Gaithersburg, MD., model H1 or H4) containing 1x TBE buffer. Samples were then loaded into the wells in 1x loading buffer (10x loading buffer; 40% (w/v) bromophenol blue, 40% (w/v) xylene cyanol, and 25% (w/v) ficoll type 400) and elecrophoresed at constant voltage. To visualize nucleic acid samples, the entire gel was immersed in a container with 50 µg/ml ethidium bromide in H_2O for 15 to 30 min. The gel was then placed on a UV box that emits at 254 nm. Nucleic acid molecules binding ethidium bromide are fluorescent at this wavelength and can be visualized by eye. A permanent record of ethidium bromide stained gels was recorded by photography using Polaroid type 55 positive-negative film with a Polaroid MP-4 camera.

Alkaline/Agarose gel electrophoresis: Agarose gels used for alkaline electrophoresis were prepared by dissolving the

appropriate amount of agarose in 270 ml of H_2O and 30 ml of 500 mM NaCl and 10 mM EDTA. The agarose was dissolved by boiling in a microwave oven. The solution was cooled to 60^0C poured in a plastic casting tray (BRL, Gaithersburg, Md.) and the desired comb was inserted. When the gel hardened, the comb was removed and the gel was submerged in an electrophoresis rig (BRL; see above) containing 30mM NaOH and 1mM EDTA, and allowed to soak for at least 0.5 hr. Samples were diluted in a buffer containing 50 mM NaOH, 1 mM EDTA, 2.5% (w/v) Ficoll type 400, 0.025% (w/v) bromocresol green and loaded onto the gel, which was electrophoresed at 45 mA overnight for 12 to 16 hrs. After electrophoresis, the gel was removed from the denaturing buffer and neutralized in 250 mM Tris-HCl, pH 7.2 for 1 hr.

Formaldehyde/Agarose gel elecrophoresis: RNA was typically electrophoresed in agarose containing formaldehyde as a denaturing agent to allow accurate separation of deproteinized mRNA that ordinarily contains a high amount of secondary structure in aqueous solution. 3.75 grams of agarose were dissolved in 181 ml H_2O by boiling in a microwave oven. 25 ml of 10x MOPS buffer (50 mM 3-[N-Morpholino] propanesulfonic acid, 25 mM sodium acetate, 5 mM EDTA, pH 7.0) and 44 ml 37% formaldehyde were mixed and heated to 60^0C. The two solutions were mixed, and poured into a gel casting tray as described above for other agarose gels. The gel was then placed in an electrophoresis apparatus (see above) in 1x MOPS buffer. Samples were loaded

in sample buffer containing 1x MOPS, 0.088% (v/v) formaldehyde, and 70% (v/v) formamide), and electrophoresed. RNA could also be visualized by ethidium bromide staining as described previously, however the gel had to be extensively soaked in H_2O to lower backround staining.

Polyacrylamide denaturing sequencing gels: 40% (w/v) acrylamide stocks were made by dissolving 38 grams of acrylamide and 2 grams of bis (N,N' methylene-bis-acrylamide) into 100 ml H_2O, filtering through a Nalgene 0.22 nm filter and storing at 4^0C in the dark. Acrylamide stocks were used for no more than 2 weeks after preparation. Generally, 8% (w/v) gels were cast by mixing 15 ml 40% acrylamide stock, 7.5 ml 10x STBE buffer (10x STBE; 0.05 M Tris, 0.04 M boric acid, 1 mM EDTA pH 8.3), 36 grams urea, and 27.5 ml H_2O together with 500 µl 10% (w/v) ammonium persulfate (APS), and 40 µl tetramethylethylenediamine (TEMED; Bio Rad Laboratories, Richmond, CA). The solution was poured into two glass plates that had been taped together and separated by 0.4 mm spacers. The gel was allowed to polymerize overnight and the following day was placed in a BRL Model SO sequencing gel apparatus containing 1X STBE buffer at the anode and the cathode. Samples were diluted in buffer containing 80% (v/v) formamide and 10 mg/ml of bromophenol blue and xylene cyanol, loaded onto the gel and electrophoresed at 40 mA , maintaining the gel temperature at 50^0C. The gel was then removed from the glass plates and soaked in a solution of 10% (v/v) acetic acid and 10% (v/v)

methanol for 0.5 hr. The gel was then transferred to a cardboard backing and dried for 1 hr. on a slab gel dryer (Model SE 1150, Hoefer Scientific Instruments, San Francisco, CA) at 80^{0}C. The gel was then autoradiographed on Kodak XAR-5 film.

SDS-PAGE Gels: Electrophoresis of proteins was based on a procedure developed by Laemmli (1970). SDS-PAGE gels were cast and run on the Protean II system (Bio-Rad Laboratories, Richmond, Ca). Acrylamide stocks were made by dissolving 29.2 grams acrylamide and 0.8 grams bis (see above) in 100 ml water to make a 30% (w/v) stock. The separating gel was made by dilution of the acrylamide stock to a final concentration of 10-12% (w/v) in 0.1% (w/v) SDS, 0.38 M Tris-HCl pH 8.8, 0.033% (w/v) APS, and 0.05% (v/v) TEMED. After polymerization of the separating gel the stacking gel was layered on top of the separating gel, and consisted of 4.5% (v/v) acrylamide, 0.1% (w/v) SDS, 0.125 M Tris-HCl, pH 6.8, 0.03% (w/v) APS, 0.3% (v/v) TEMED. Samples were then loaded onto the gel (protein lysates prepared as described above) and electrophoresed at 20 mA through the stacker and 30 mA through the separating gel. The electrophoresis buffer was 0.025 M Tris-glycine, pH 8.5, 0.1% (w/v) SDS.

Northern Blots

After formaldehyde gel electrophoresis of RNA samples, the gel was soaked in 20X SSC (3 M NaCl, 3.3 M sodium citrate, pH 7.0) for 1 hr. Nytran sheets were then cut to the size of

the gel and the gel was placed in a standard wick apparatus so that the RNA could transfer from the gel by capillary action onto the nylon membrane overnight. The nytran paper was then baked for 2 hrs. at 80^0C in a vacuum oven. The blot was then prehybridized for 4-8 hrs in a solution containing 50% (v/v) formamide, 4X Denhardts (1% (w/v) ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA pentax fraction V), 5X SSC, 0.05 mM KPO_4, pH 7.0, and 200 µg/ml salmon sperm DNA at 42^0C. The appropriate radioactive probe was then added and hybridized for 12-48 hrs., and then the blots were washed. Blots were first washed two times at room temperature in 2X SSC, 0.5% (w/v) SDS, 50 mM KPO_4, pH 7.0, and then two 15 minute washes in 0.1X SSC, 0.5% (w/v) SDS, 50 mM KPO_4 pH 7.0. The blots were then air dried, wrapped in Saran Wrap and autoradiographed on X-Ray film (Kodak XAR-5, in cassettes with enhancing screens at -70^0C).

Purification of DNA fragments

DNA fragments were purified by visualizing their mobility on neutral agarose gels after electrophoresis as described previously. Appropriate bands were excised with a razor blade, and the agarose pieces were placed in an analytical electroeluter (model UEA, International Biotechnologies, Inc., New Haven, CT), following the manufacturer's protocol. Briefly, the apparatus was filled with 1X TBE and the V-shaped wells with 7.5 M ammonium acetate containing bromophenol blue. Electrophoresis was carried out for 1 hr.

at 100 volts. The ammonium acetate which contained the DNA was removed with a tuberculin syringe and precipitated in 2 volumes isopropanol. The DNA was pelleted at 12,000 r.p.m. in a microfuge for 20 minutes and resuspended in TE. A small amount of the total sample was tested on agarose gel electrophoresis and ethidium staining to determine percent recovery and purity.

Molecular cloning of DNA fragments

The strategy for generation of recombinant plasmids varied greatly. The following is an explanation of the general chemical manipulations used to modify DNA appropriately for insertion into the desired vector. Several buffers were used to perform these manipulations and are listed here:

10x Kinase buffer: 0.5 M Tris-Cl (pH 7.6), 0.1 M $MgCl_2$, 50 mM dithiothreitol (DTT), 1 mM spermidine, 1 mM EDTA.

10x Fill-in buffer: 0.5 M Tris-Cl (pH 7.5), 0.1 M $MgCl_2$, 10 mM DTT.

10x Calf Intestine phosphatase buffer(CIP): 0.5 M Tris-HCl pH 9, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine.

10x ligation buffer: 0.66 M Tris-HCl pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 0.3mM ATP, 1 mM spermidine-HCl, 1 mM hexaminecobalt chloride, 200 µg/ml BSA.

In some cases, DNA was generated with restriction enzymes that resulted in ends that were compatible with the vector, in which case no manipulation was necessary. If necessary, DNA fragments that had protruding 5' ends were changed to a

blunt-ended fragment. The typical reaction conditions were 1 µg DNA, 2 mM all 4 dNTPs, 2.5 µl 10x nick-translation buffer, and 2 units Klenow fragment in a volume of 25 µl for 0.5 hr. Additionally, linkers could be ligated at this time by adding a 20-fold molar excess of linkers over total 5' ends of target DNA and 2 units T4 DNA ligase at 15^0C for 12 hr. To obtain single copies of linkers on each end, the reaction was then cut with the appropriate restriction enzyme and excess linkers were purified away by agarose electrophoresis of the DNA fragment and electroelution. In these studies two linkers were used, BamH I and Bgl II. The linkers were purchased from Pharmacia (Piscataway, NJ), and the sequences were 5'-d(CCCGGATCCGGG)-3' for the BamH I linkers and 5'-d(CAGATCTG)-3' for the Bgl II linkers. To prepare linkers for ligation, they were first 5' phosphorylated by the kinase reaction described below (see radiolabeling of DNA fragments) only non-radioactive ATP was used. The linkers were then annealed into a double-stranded form by heating to 90^0C for 5 min. and cooling to room temperature (RT) slowly over the course of about an hour. Linkers were then stored at 4^0C.

Vector DNA termini were dephosphorylated by treating 10 pmol ends with 0.1 units calf intestine phosphatase 5 µl 10x CIP buffer in a volume of 50 µl. The reaction was terminated by phenol/chloroform extraction and DNA was recovered by ethanol precipitation. Dephosphorylation of vector ends results in negligible amounts of vector ligation upon itself.

Typical ligation conditions were a molar ratio of 3:1 insert DNA to vector DNA, 2 µl 10x ligation buffer, and 2 units T4 DNA ligase, in a volume of 20 µl for 12 hrs at 15^{0}C. Typically, 1% of the ligation reaction was added to competent bacteria for transformation.

Bacterial transformation

Bacteria were made competent by the standard Hanahan method, or commercially available bacteria were used (BRL, Gaithersburg, Md., made by the Hanahan method) (Hanahan, 1983). In all of these studies the DH5α strain of bacteria was employed. 1 µl of ligation reaction was added to 100 µl competent bacteria for 0.5 hr. on ice. The cells were then subjected to heat shock at 42^{0}C for 2 minutes and placed again on ice. 400 µl of S.O.C. medium (2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM each $MgCl_2$+$MgSO_4$, 20 mM glucose) was added and the cells were incubated on a rotating wheel at 37^{0}C for 2 hrs. The bacteria were spun at low speed in a microfuge and resuspended in 100 µl S.O.C. This solution was then spread onto sterile bacterial plates containing the antibiotic of choice (usually ampicillin since most of the vectors in this study contained an ampicillin resistance gene). In some cases plates contained XGal (2% v/v) and IPTG (25 mg/ml) for visualization of bacterial colonies positive (blue colony) or negative (white colony) for the production of β-galactosidase

(β-gal). Plates were incubated overnight at 37°C in a non-CO_2 incubator.

Identification of recombinant plasmids

Bacterial colonies were screened by picking white colonies since inactivation of the β-gal gene in the multiple cloning site occured by insertion of the desired DNA fragment. In cases where vectors did not contain this screening system all colonies had to be picked. Colonies were replica-plated on LB agar plates containing ampicillin, with one of the plates containing a Biodyne membrane placed directly on the agar surface. The plates were incubated overnight to allow growth. The following day, master plates were placed at 4°C and the plates with the biodyne membranes were placed in a solution of 0.5 M NaOH, 1.5 M NaCl. The Biodyne membrane was then transfered through two 5 minute washes of Tris-HCl pH 7.5 and 1.5 M NaCl. The final wash was for 30 seconds in 2X SSC, and membranes were then baked at 80°C for 2 hrs. in a vacuum oven. Detection of desired colonies was by Southern hybridization with the appropriate radiolabeled DNA fragment complementary to the DNA insert of interest, and autoradiography (see Southern hybridization methodology). Once "positive" colonies were identified by autoradiography, bacteria from the master plate were grown in small cultures and DNA was prepared from these cultures by the rapid method described by Maniatis et al. (1982). The DNA was cleaved by restriction enzymes, electrophoresed on agarose gels and

visualized by staining with ethidium bromide to confirm the orientation and presence of DNA insert. If large amounts of recombinant DNA were required, bacteria were grown in liter quantities and DNA was purified by CsCl-ethidium bromide centrifugation as described by Maniatis et al. (1982).

Southern Blots

The procedure for blotting of DNA from agarose gels is a modification from the procedure described by Southern (1975). After ethidium staining and photography, agarose gels were treated for 1 hr. in a solution of 0.5 M NaOH and 0.2 M NaCl. The gel was neutralized by treatment of the gel for 1 hour in 1.0 M Tris-HCL, pH 7.5 and 0.5 M NaCl. The DNA was transferred in 6X SSC overnight to a Biodyne membrane. Hybridization of labeled DNA to these membranes was carried out by one of two methods. If double-stranded DNA probes were used (labeled by the random hexamer primer method or nick-translation), blots were prehybridized (4 hrs) and hybridized (12 hrs) in 30% (v/v) formamide, 6X SSC, 1X Denhardt's, 0.5% (w/v) SDS at 50^0C. They were then washed at room temperature in 2X SSC , 0.5% (w/v) SDS and two washes in 2X SSC at 50^0C. The blots were then wrapped in Saran Wrap and autoradiographed at -70^0C in casettes with enhancing screens, using Kodak XAR-5 X-ray film. If oligonucleotide probes were used (labeled by T4 DNA Kinase), blots were prehybridized (2 hrs.) and hybridized (2 hrs.) in 6X SSC, 50 mM $NaPO_4$, 5X Denhardt's, 100 µg/ml salmon sperm DNA. Three 5

minute washes were carried out in 6X SSC, 50 mM $NaPO_4$, 1% (w/v) SDS. The temperature for all treatments is at the T_h for the oligonucleotide, calculated as: $T_h=T_d-5^0C$ where T_d = 4(G+C)+2(A+T). Autoradiography was carried out as described above.

Plasmids

The plasmid vectors used in this study are all either commercially available or are well documented in the literature. Bluescript SK+ (Stratagene, La Jolla, CA), pUC 19 (BRL, Gaithersburg, Md.) and pGEM 3Z (Promega Biotech, Madison, WI), were all purchased from the manufacturer. pSC11 was generously donated by Dr. Bernard Moss and the plasmid map has been published. pA10CAT2, pCAT3M, and pSV2 were all generously provided by Dr. George Khoury (NIH), and a map of important sequences and restriction sites is shown in the appendix. Schematic representations of recombinant plasmids generated for use in this study and pertinent data regarding orientation and confirmation of DNA insertion are shown in the Appendix.

Oligonucleotides used in this study

Oligonucleotides were synthesized by M.F. Flora in the Department of Microbiology, USUHS, using B cyanoethyl diester phosphoramidite chemistry on an Applied Biosystems model 380A synthesizer (Applied Biosystems Inc., La Jolla, CA).

Oligonucleotides used in these studies are listed by name, sequence, and complementary gene:

OPL1	5'TTGAGATTCAGTCGATAATT3'	gpV
OPL5	5'CCTTGAAGATCAAAGCGTTG3'	gpIV
OPL15	5'ATCCAGTGATTTTTTTCTCC3'	CAT

Oligonucleotides were stored in H_2O at -70^0C, at a concentration of 1 mg/ml.

Western Blots

After electrophoresis of proteins on SDS-PAGE gels, the gels were removed from the glass plates and washed in a buffer (WB1) containing 0.012 M Tris-glycine, pH 8.5 with 20% (v/v) methanol. The proteins were transfered to 0.45 μm nitrocellulose, in an electrotransfer apparatus (Model TE 52, Hoefer Scientific Instruments) according to the manufacturer's protocol. Typically, proteins were transferred overnight at 0.5 amps in WB1 buffer. Following transfer, the blots were washed in a buffer (WB2) containing 10% (w/v) Carnation instant milk, 0.15 M NaCl, 50 mM Tris-HCl, 0.05% (v/v) Tween 20, 10 mM EDTA, 0.02% (w/v) Na azide, pH 7.6. The blots were then incubated at RT with WB2 and 1% (w/v) milk, with a 1/100 dilution of antibody (see "preparation of antibodies") for 2 hours. The excess antibody was then washed off by several rinses in WB2 in 1% (w/v) milk, and the blot was incubated at room temperature in WB2, 1% (w/v) milk with a 1/1000 dilution of ^{125}I-labeled staphlococcus protein A (NEN Research Products, specific

activity 120 μCi/ml) for 2 hrs. at room temperature. The blots were then washed in WB2, dryed, wrapped in Saran Wrap, and autoradiographed (Kodak XAR-5) film.

Immune precipitations

VZV-infected cells at approximately 50% CPE were transferred into glucose-free media that contained ^{14}C-glucosamine, and labeled for 18-24 hrs. For pulse-chase experiments, VZV-infected cells at 50% CPE were transferred into medium containing ^{35}S-methionine for 15 min., and then washed. Cold methionine containing medium was replaced for 4 hrs. at which time the cells were harvested. VZV-infected cell monolayers were lysed in a buffer containing 1% (v/v) NP40, 1% (w/v) deoxycholate, 0.1 mM PMSF, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 1 mM DTT, 0.15 M NaCl. Usually cells were grown in 75 cm^2 flasks and lysed with 1 ml lysis buffer. The extracts were then sonicated, and spun at 4^0C in a Ti50 rotor at 50,000 r.p.m. for 1 hr. The supernantant was saved and 100 μl was mixed with 10 μl antiserum, and incubated overnight at 4^0C. Subsequently, 100 μl of 33% (v/v) protein A sepharose beads were added and incubated for 1-2 hrs. at 4^0C. The beads were washed 5x, the supernatant removed, and 200 μl sample buffer (see protein lysate procedure) was added. Samples were run as described for SDS-PAGE gels. The gels were then fixed in 10% (v/v) methanol and 10% (v/v) acetic acid for 1 hr., dried, and autoradiographed.

Restriction enzyme digestion of DNA

DNA was stored in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and diluted in buffers supplied by the manufacturer for digestion by restriction enzymes. The amounts of enzyme used, temperature and time of digestion were as recommended in the manufacturer's protocol. Once digestion was complete, the reaction was stopped by addition of 10x loading buffer at a 1/10 dilution (described under agarose gel electrophoresis). Samples were then electrophoresed on agarose gels as described above. In some cases, additional manipulations (such as other restriction enzyme digestions or other modifications) of DNA were required, and the reaction was stopped by heat inactivation of the enzyme or phenol/chloroform extraction prior to subsequent procedures.

Radiolabeling of DNA fragments

DNA was labeled to high specific activity by several methods. Oligonucleotides and DNA fragments dephosphorylated at their 5' ends were labeled at their 5' ends by T4 polynucleotide kinase. Reaction conditions were set up as described in Maniatis et al.(1982). DNA containing recessed 3' ends was labeled at the 3' ends by the Klenow fill-in reaction described in detail in "Guide to Molecular Cloning Techniques" (Berger and Kimmel, 1987). DNA fragments, or whole plasmid DNA was labeled by the nick-translation method using a nick-translation kit (BRL, Gaithersburg, Md.), following the manufacturer's protocol. Finally, DNA

fragments were also labeled by a random hexamer labeling kit (Boeringer Manheim, Indianapolis, IN) according to the suggested protocol.

Cloramphenicol acetyl-transferase assays

Plasmids used in chloramphenicol acetyl-transferase (CAT) assays were obtained or made as described previously. *DNA transfections*: Initially, DNA transfections were performed using the $CaPO_4$ precipitation method as described previously (Graham and Van der Eb, 1973). Later, the DEAE-Dextran method was used according to the detailed protocol in "Protocols in Molecular Biology" (Ausubel et al., 1987). Approximately 10 µg of target plasmid was routinely used to transfect 10 cm tissue culture plates. Except where indicated, all transfections were carried out using human foreskin fibroblast cells. To infect transfected cells with VZV, infected monolayers of VZV were trypsinized, and added to transfected cells at a ratio of 1 to 10 infected to uninfected cells for virus transactivating experiments. When co-transfection of target plasmids was done, activator plasmids were added at 5 µg per 10 cm tissue culture plate. *Preparation of cell extracts* : After transfection, harvesting the cells and performing CAT assays was carried out by the method of Gorman et al. (1982). We used equal amounts of protein as determined by Bradford (1976), for each extract in a given experiment. To quantitate levels of acetylation, acetylated and unacetylated spots were localized

by autoradiography and excised from thin layer chromatography plates and counted in nonaqueous scintillation fluid on a Beckman LS9000 scintillation spectrometer to obtain the percentage of acetylation.

Generation of antisera

Human anti-VZV antisera were obtained by bleeding patients 3-6 weeks after recovery from an attack of zoster. The blood was allowed to coagulate, the clot was spun out, and the resulting serum was aliquoted and stored at -20^0C.

Anti-gpIV and gpV antisera were generated by subcutaneous inoculation of $>1x10^7$ PFU vaccinia recombinants into rabbits. The rabbits were bled starting 2 weeks post inoculation and serum tested for reactivity against VZV-infected cell lysates in western blot assays. Once it was determined that the rabbit was making antibodies to the VZV protein expressed by the vaccinia recombinant, the rabbits were "production bled" and then sacrificed.

Generation of Vaccinia recombinants

The shuttle vector pSC11 with an AccI/AccI fragment which contains the VZV gene 67 ORF in the proper orientation to be expressed by the vaccinia p7.5 late promoter was isolated as described in the molecular cloning section. The recombinant plasmid DNA was then purified by CsCl-ethidium bromide gradient centrifugation. CV-1 cells infected with wild-type vaccinia virus were then transfected with calcium phosphate-

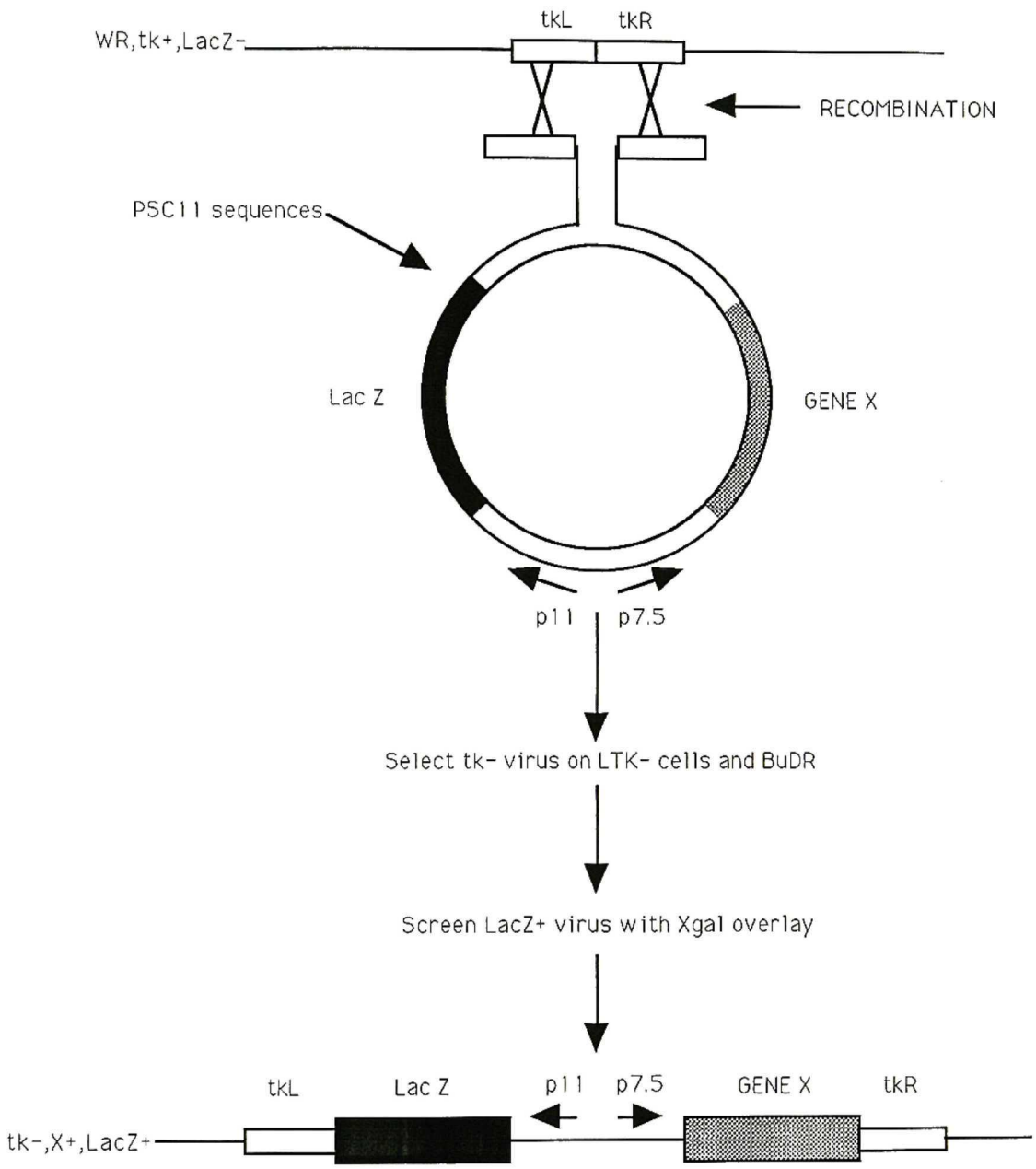
precipitated DNA from pSC11 gpIV recombinant clones. Recombination of the thymidine kinase (TK) sequences in pSC11 with the wild-type vaccinia virus TK sequences inactivates the TK gene resulting in a TK^- virus. TK^- virus was then selected by growth on TK^- 143 cells in the prescence of BUdR and the resulting recombinants were assayed for blue plaque production in the presence of X-Gal. This is shown in diagramatically in figure 6. At least three rounds of plaque purification were used for each isolate. Verification that recombinant viruses contained the entire VZV gene inserts, was accomplished by Southern blotting of the recombinant vaccinia virus DNA. Vaccinia virus DNA was prepared as described previously, and cut with BamH I/EcoR I and southern blotted as described above, using the AccI/AccI fragment containing the VZV gene 67 DNA ORF as a probe (see appendix). Recombinant vaccinia viruses expressing the VZV gpI (ORF68) and gPV (ORF14) gene products were prepared in a similar manner except that the gpI gene was encoded in a Bgl I/Sma I fragment and the gpV gene was in a BstN I/Alu I fragment. (see Appendix for all pSC11 cloning schemes)

Virus neutralization assays

HFF cells were infected with VZV-infected cells at a 20:1 ratio. After approximately 10% CPE had developed, the cells were typsinized and reseeded in the same flask. 24 hrs. later, this step was repeated with addition of an equal number of uninfected cells. Cells were harvested 24-48 hrs.

FIGURE 6

Schematic Diagram for generation of Vaccinia recombinants. This figure shows the general procedure for generation of a vaccinia recombinant. The top of the figure shows a plasmid containing two vaccinia promoters, one driving β-galactosidase (p11) and the other driving a foreign gene (p7.5). Construction of plasmids with VZV glycoprotein genes and other characterisitics of the pSC11 shuttle vector are described in the Appendix. The diagram shows regions of the vaccinia tk gene in the plasmid recombining with the vaccinia tk gene in the wild-type virus to form a tk^- variant. Tk^- viral mutants are selected for the ability to grow in the presence of BUdR. To screen for recombinants that contain the foreign gene, versus spontaneous tk^- viral mutants, plaque overlays containing X-gal allow visual identification of blue plaques i.e.; those recombinants that also received the β-gal gene. The bottom of the figure shows the relationship of important sequences in a successful recombinant vaccinia virus.


WR,tk+,LacZ-
tkL
tkR
RECOMBINATION
PSC11 sequences
Lac Z
GENE X
p11
p7.5
Select tk- virus on LTK- cells and BuDR
Screen LacZ+ virus with Xgal overlay
tkL
Lac Z
p11
p7.5
GENE X
tkR
tk-,X+,LacZ+

later and ultrasonically disrupted in MEM with 20% (v/v) fetal calf serum diluted in phosphate buffered saline. After incubation for 2 hrs. the mixture was then applied to subconfluent HFF monolayers and plaques counted after 4-6 days.

Indirect Immunofluorescence

Subconfluent monolayers of HFF cells were grown on glass cover slips and infected with VZV Scott as described above. Two to four days later, cells were washed three times in ice-cold PBS and fixed in acetone at -20^{0}C for 10 min. Cover slips were then rehydrated in PBS containing 10% (v/v) FCS for 15 min. at room temperature. Following this, they were incubated with an appropriate dilution of antibody for 1 hr. and then washed three times with PBS. Cells were then exposed to goat anti-rabbit immunoglobulin G conjugated with rhodamine (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 hr. and washed three times with PBS. The coverslips were then mounted in 50% (v/v) glycerol and viewed with a phase-contrast fluorescence microscope (Carl Zeiss Inc., Thornwood, NJ).

S1 Nuclease Analysis

Structural analysis of gpV transcripts was performed by using the S1 digestion procedure of Berk and Sharp (1978) with some modifications. 5' or 3' end labeled probes were hybridized at 45^{0}C to appropriate amounts of RNA for 16 hrs.

The reaction mixture was then digested with 10 to 100 units of S1 nuclease (BRL) for 30 minutes. The digestion products were then electrophoresed on neutral or alkaline agarose gels and detected by autoradiography. In some cases, digestion products were resolved on polyacrylamide denaturing sequencing gels. A schematic diagram of the S1 procedure is shown in figure 7.

Primer Extension analysis

Primer extensions were performed essentially as described by Inoue and Cech. (1985). 100 ng of oligonucleotide was 5' end labeled and purified through a G15 spin column. 10-20 ng of labeled oligo was then incubated with 15-25 ug of RNA in 40 mM PIPES, 1 mM EDTA, 0.2% (w/v)SDS and 0.4 M NaCl, at 37^0C for 2 hrs. The reaction mixture was then precipitated in ethanol and the pellet was resuspended in 50 mM Tris-HCl pH 8.3, 6 mM $MgCL_2$, 40 mM KCl, and 10 mM DTT. The reaction mixture was then brought to 0.5 mM dNTPs, 20 units reverse transcriptase, and incubated at 37^0C for 1 hr. The reaction was terminated by phenol/chloroform extraction followed by ethanol precipitation. Primer extension products were then electrophoresed on polyacrylamide sequencing gels and visualized by autoradiography. A schematic diagram of the primer extension procedure is shown in Figure 8.

FIGURE 7

Schematic diagram of the S1 nuclease procedure. The figure shows two RNA/DNA heteroduplexes formed by hybridizing a uniquely end-labeled probe fragment with an RNA preparation of interest. Digestion of the heteroduplexes with S1 nuclease yields two distinct products. The products in these hypothetical experiments are slightly different in size. Under neutral conditions, the RNA/DNA heteroduplex molecules remain together and the products from the S1 procedure will migrate according to their size and can be detected by autoradiography after electrophoresis. However, the product on the left has a "nick" in the DNA fragment due to the looping out of complimentary DNA not present in the spliced transcript. Electrophoresis of these products under alkaline conditions will denature the heteroduplexes. Only the DNA probe fragment will be detected by autoradiography, and the probe hybridizing to the spliced RNA will migrate much faster because of its smaller size.

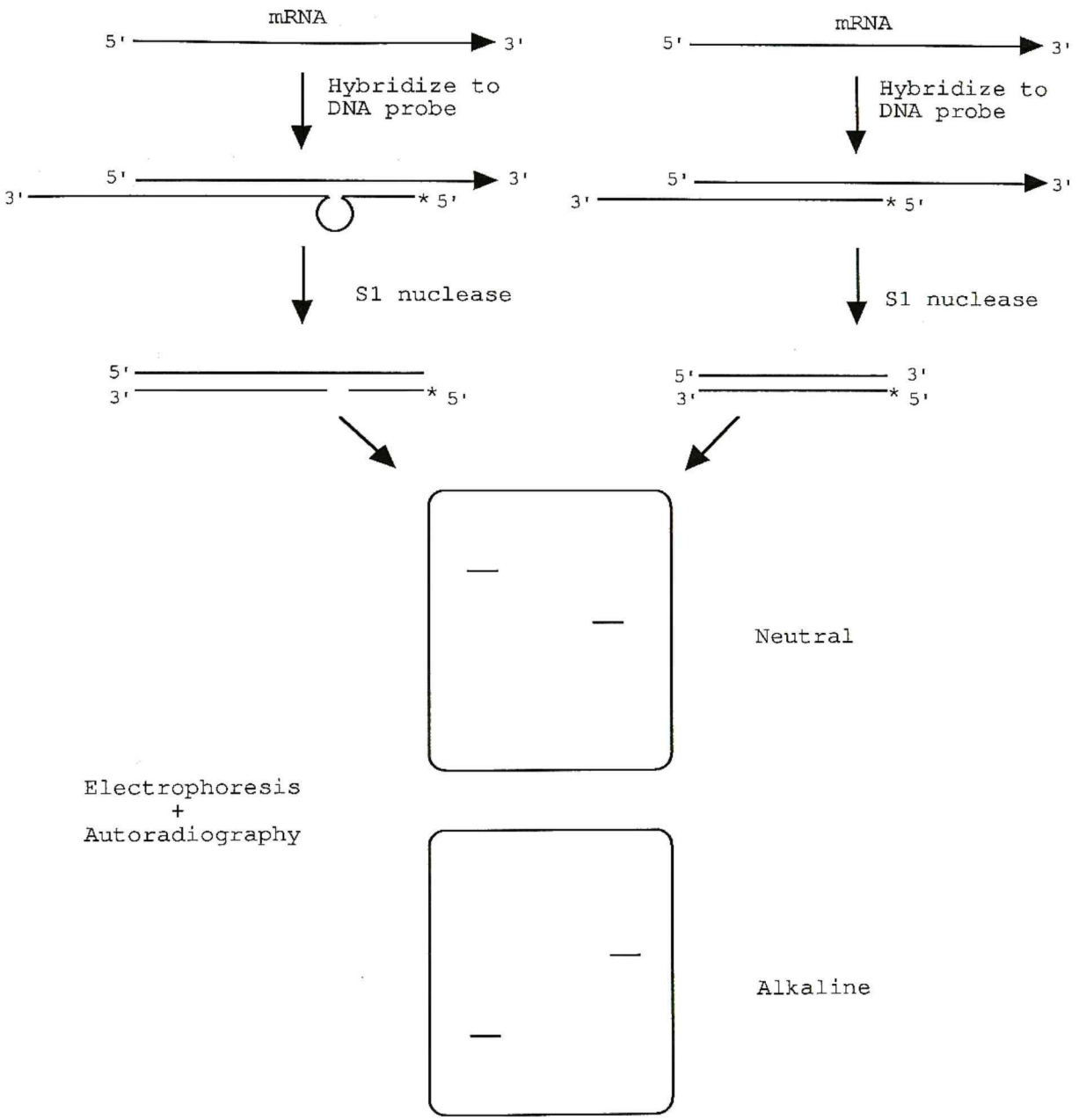
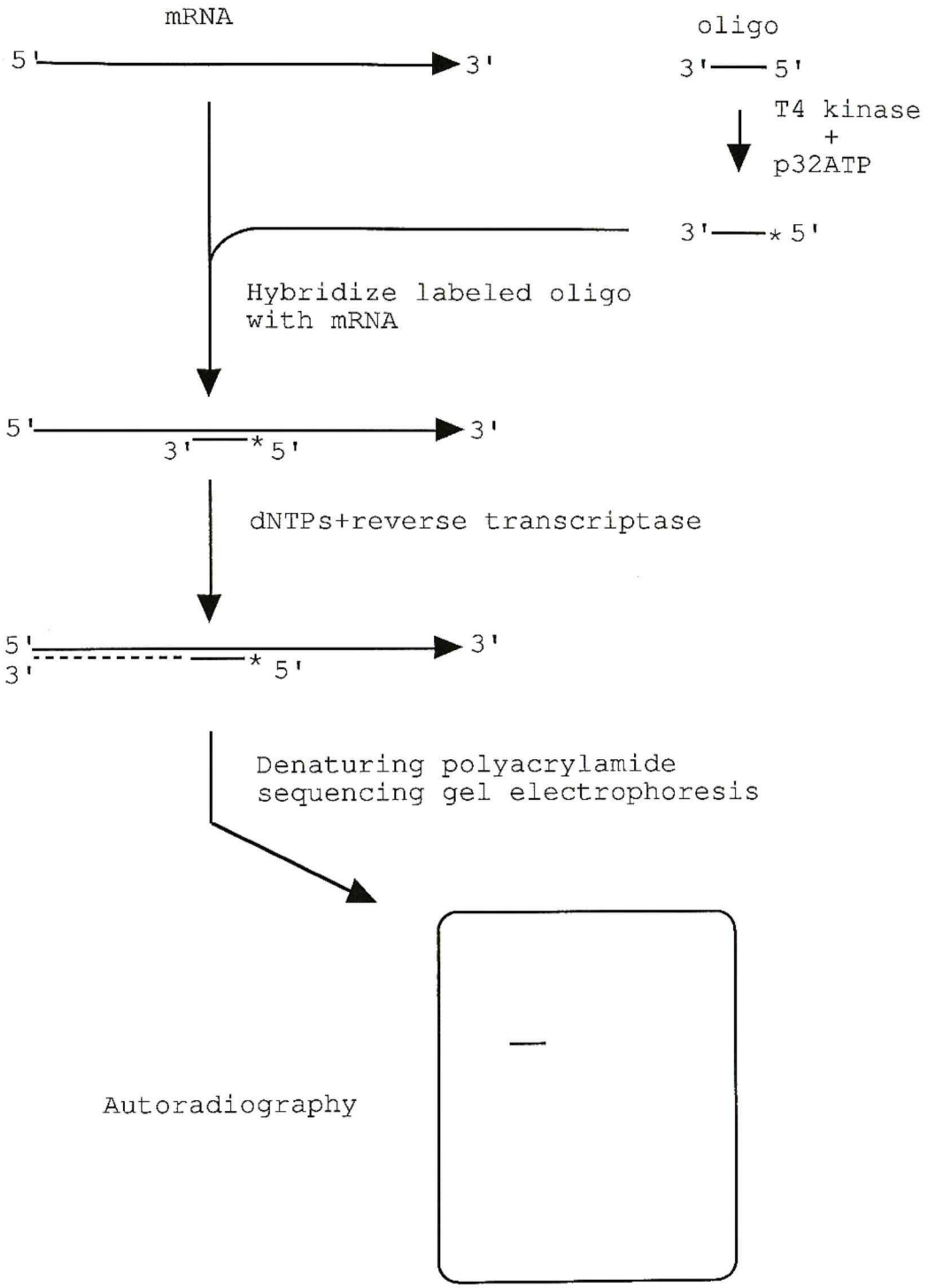

mRNA
5'
3'
Hybridize to
DNA probe
3'
5'
* 5'
S1 nuclease
Neutral
Electrophoresis
+
Autoradiography
Alkaline

FIGURE 8

Schematic of the Primer Extension technique. The top right of the figure shows an oligonucleotide (oligo) that is labeled by conventional methods (see "kinasing" under molecular cloning techniques). The oligo is made such that it hybridizes to the RNA molecule of interest, and can be used as a primer for reverse transcriptase. Addition of reverse transcriptase and nucleotides, results in synthesis of a single-stranded DNA copy extending to the 5' end of the RNA molecule. The "cDNA" molecule can be resolved on a denaturing polyacrylamide sequencing gel and its size determined based on migration of standard markers. The location of the oligo sequence in the gene can then allow the investigator to determine the 5' location of transcript initiation.


mRNA
5'
3'
oligo
3'
5'
T4 kinase
+
p32ATP
3'
*
5'
Hybridize labeled oligo
with mRNA
5'
3'
3'
*
5'
dNTPs+reverse transcriptase
5'
3'
3'
*
5'
Denaturing polyacrylamide
sequencing gel electrophoresis
Autoradiography

Radioiodination of antibodies using the Chloramine-T method

Radioiodination of Human IgG and Fab'$_2$ molecules (purchased from Cappel laboratories, Belgium) was performed as according to Maniatis et al. (1982). Briefly, 70 µg of antibody was brought to 50 µl in PBS pH 7.2, then 500 µCi of carrier-free ^{125}I were added. 15 µl of Chloramine-T (2 µg/ml) was added for 1 min., at which time the reaction was stopped with 50 µl stop buffer (0.1 M sodium phosphate buffer pH 7.2, 10 mg/ml tyrosine, 2 mg/ml sodium metabisulfite, 10% (w/v) glycerol and 0.1% (w/v) xylene cyanol). The solution was then added to a G-25 column, and 100 µl fractions were collected from the eluate. Small amounts from each fraction were counted, and the fractions containing most of the counts were pooled. The specific activity was approximately 1x10^6 cpm/µg protein.

Assay for binding of ^{125}I-labeled antibodies to cells

Radioiodinated IgG Fc or Fab'2 was diluted in PBS containing 2.5% (v/v) fetal calf serum, placed on infected or uninfected cells, and allowed to incubate for 2 hrs. The cells were washed two times with PBS containing 2.5% (v/v) FCS and solubilized with PBS containing 0.5% (w/v) SDS. The extracts were counted using aqueous scintillation fluid and a scintillation counter.

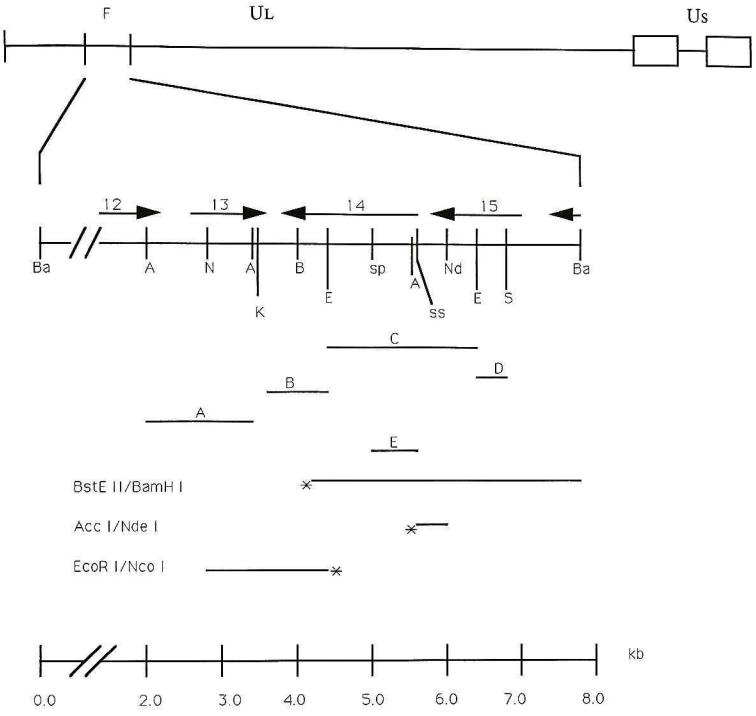
The main aim of work described in this dissertation was the detailed transcriptional analysis of two VZV glycoproteins. Having accomplished this, we began to focus on some interesting questions about VZV gene expression that arose from this data. Finally, we addressed some fundamental issues involving the glycoprotein products themselves and some of their possible activities.

Transcript analysis of gene 14 in Scott and Oka

Northern blot analysis and quantitation of RNA abundance: Previous northern hybridization studies using single stranded M13 probes suggested that 3 major transcripts of 2.5kb, 1.95kb, and 1.6kb map to the EcoR I P fragment which spans parts of gene 14 and gene 15 of VZV (Kinchington et al., 1986; Ostrove et al., 1985). To determine the origin and structure of these transcripts, and to gather transcript information on the genes surrounding gene 14, a number of probes (figure 9, probes A-E) generated from the BamH I F fragment were hybridized to RNA from VZV-infected cells in northern hybridization experiments. All assays were carried out with poly(A)$^+$ RNA; control blots using total VZV RNA were identical to poly(A)$^+$ RNA blots. Experiments comparing poly(A)$^+$ to poly(A)$^-$ RNA fractions show that the poly(A)$^-$ fractions contained no detectable or barely detectable virus-

FIGURE 9

Summary diagram depicting the probes used for identification of gpV RNAs. A schematic of the entire VZV genome is shown at the top of the figure with the approximate location of the BamH I F fragment. The BamH I F fragment is expanded below, showing the size and location of four ORFs - 12, 13, 14, and 15, (solid lines with arrows). Various restriction enzyme sites are shown by vertical lines; the abreviations are: A; Acc I, Ba; BamH I, B; BstE II, E; EcoR I, K; Kpn I, N; Nco I, S; Sma I, sp; Sph I, ss; Sst II. The horizontal lines labeled A-E show the size and location of probes used for northern hybridization studies. The horizontal lines with an asterisks were probes used in S1 protection analysis. Also shown at the bottom of the figure is an approximate scale in kilobases.


F
UL
Us
12
13
14
15
Ba
A
N
A
B
sp
A
Nd
Ba
K
E
ss
E
S
C
D
B
A
E
BstE II/BamH I
Acc I/Nde I
EcoR I/Nco I
kb
0.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0

specific RNA (figure 10). Thus, at least for this portion of the viral genome, all viral RNAs appear to be polyadenylated.

Northern blots, with the EcoR I P fragment (figure 9, probe C) yield results which confirm our previous studies with strain Scott, except that we now consider the 1.6-1.7kb transcript to be 1.5kb (figure 11a, panel C, lane S, also figure 10). Strain Oka, as expected, produces a 1.8kb and a 2.3kb transcript, correlating with the 168bp missing from its ORF (figure 11a, panel C, lane O), as well as the 1.5kb RNA. We suggest that the 1.5kb species originates from gene 15, since another probe (figure 9, probe D) that begins >500bp upstream of the gene 14 initiation codon also detects it (figure 11a, panel D, lanes S and O). It also fits with the size of the gene 15 ORF (1218bp).

The data further suggest that there are two 2.5kb transcripts in the gene 14/15 region, one originating from gene 14 and encoding its sequences (this is the 2.3kb transcript found in Oka), and a 2.5kb read-through transcript encoding gene 15 and gene 14 (total ORF size 2442bp) that is present in and is identical for both strains (figure 11a, panel D, lanes S and O). This hypothesis is supported by data from a third probe, with no gene 15 sequences, (figure 9, probe B) that fails to detect a 2.5kb transcript in strain Oka, but detects the 2.3kb transcript, and detects a 2.5kb transcript in Scott that is relatively less abundant than that apparent with the EcoR I P probe (figure 11a, panel B, lanes S and O). Probe B also detects two additional RNAs, of

FIGURE 10

Northern Blot analysis of VZV poly(A)$^+$ and poly(A)$^-$ RNA. RNA from uninfected and VZV infected cells was separated into poly(A)$^+$ and poly(A)$^-$ fractions by oligo (dT) cellulose chromatography and subjected to denaturing formaldehyde gel electrophoresis. The RNA was then transferred to Nytran, and detected by hybridization to radioactive DNA probes. A) A northern blot probed with EcoR I P on uninfected (U), Scott (S), and Oka (O) RNA. Each fraction of RNA is labeled as A$^-$ poly(A)$^-$, and A$^+$ poly(A)$^+$. The numbers on the right side of the diagram are sizes of transcripts in kilobases. B) An ethidium bromide stain of the formaldehyde agarose gel prior to transfer and nothern hybridization shown in panel A. Lanes 1-6 correlate with lanes 1-6 in panel A. The numbers on the right side of the diagram are 28S and 18S ribosomal RNA.

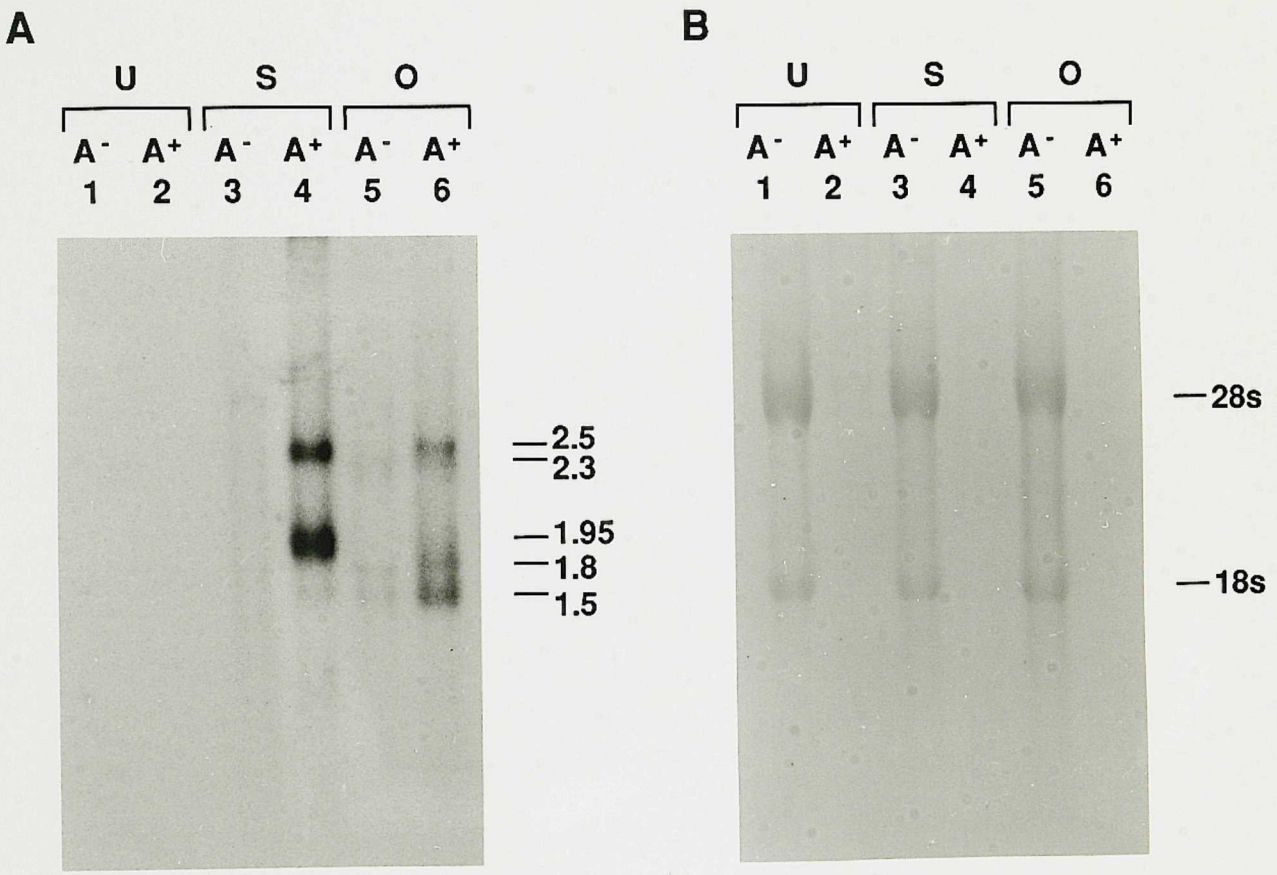
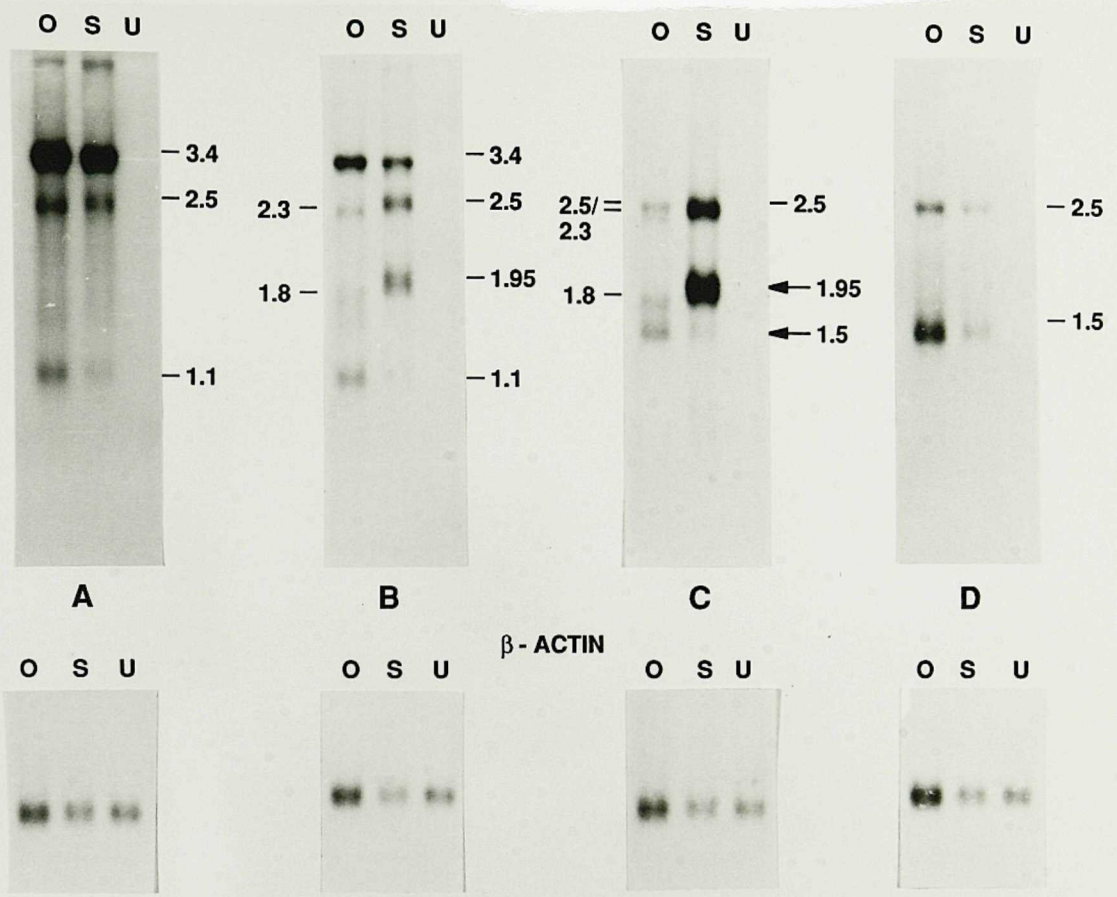

A
U
S
O
A⁻ A⁺ A⁻ A⁺ A⁻ A⁺
1 2 3 4 5 6
2.5
2.3
1.95
1.8
1.5
B
U
S
O
A⁻ A⁺ A⁻ A⁺ A⁻ A⁺
1 2 3 4 5 6
28s
18s

FIGURE 11

Northern blot analysis of VZV RNA. Total cellular RNA was harvested from VZV-infected HFF cells and the poly(A)$^+$ RNA was selected by oligo (dT) cellulose chromatography and subjected to denaturing formaldehyde gel electrophoresis. The RNA was transferred to Nytran and detected by hybridization to DNA probes. (A) Panels A-D were probed with probes A-D (see fig. 9) respectively, while panels below are the same blots stripped and reprobed with a β-actin specific probe. lane U; uninfected RNA, lane S; VZV Scott-infected cell RNA, lane O; Oka-infected cell RNA. (B) Viral transcripts detected by hybridization to probe E. Lane 1; uninfected RNA, lane 2; VZV Scott-infected cell RNA; lane 3; VZV Oka-infected cell RNA. The numbers on either side of all the panels are sizes of transcripts in kilobases.

A



B

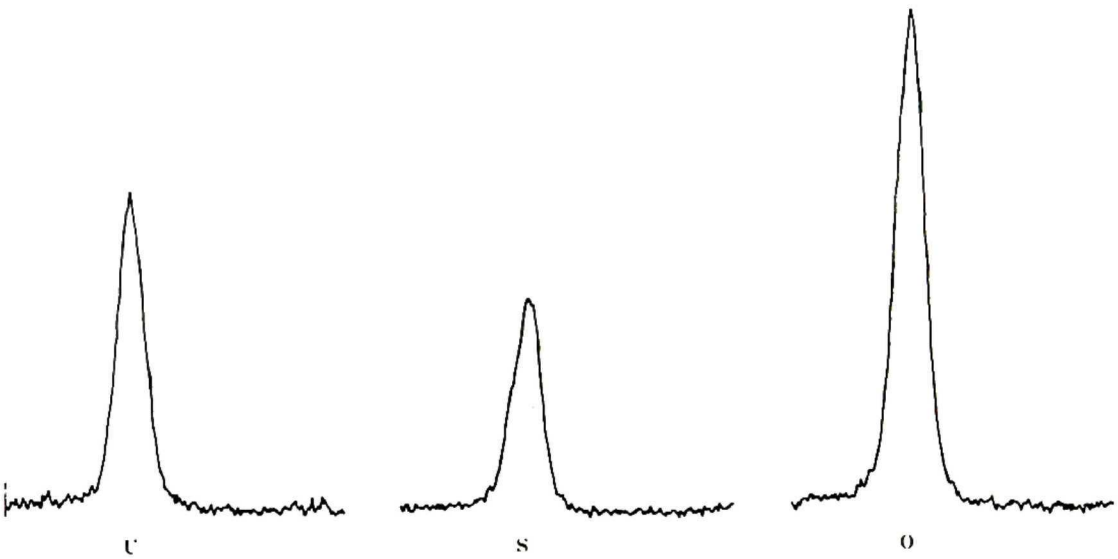


3.4kb and 1.1kb; it is likely that the 1.1kb transcript encodes gene 13 sequences (903 bp) and that the 3.4kb RNA is a readthrough transcript originating from gene 12 (1983bp). Probe A, wholly within genes 12 and 13, (figure 9), fails to detect any of the proposed gene 14 transcripts, and recognizes an additional 2.5kb species in both Scott and Oka; this is probably a (the) gene 12 transcript (1983bp) (figure 11, panel A, lanes S and O). Finally, to confirm the identification of gene 14 transcripts, we used a gene 14-specific probe (figure 9, probe E), and were able to detect only the 2.5 and 1.95kb transcripts in Scott and the 2.3 and 1.80kb transcripts in Oka strains (figure 11b).

To quantitate these RNAs, densitometric readings on autoradiograms from northern blots were taken (e.g. from figure 11a and b) and areas under peaks were measured using a Hewlett Packard 9874A Digitizer. All blots were then stripped and reacted with a β-actin probe to act as an internal control (see figure 11a). An example of the densitometric tracings is shown in figure 12. After normalization to β-actin, it was clear that viral messages, with the exception of the 1.95/1.8kb and 2.5/2.3kb transcripts, are equal in abundance for Oka and Scott. However, the ratio of transcript abundance (Scott:Oka) for the 2.5kb/2.3kb message is approximately 15, and for the 1.95kb/1.8kb message is about 26. The results of these comparisons are shown in table 1.

FIGURE 12

Densitometry tracings of VZV northern blot autoradiograms. Two autoradiograms with bands in the linear range of exposure were scanned by a Biorad scanning densitometer. The results were recorded on an LKB stripchart recorder. Peaks are labeled according to the band they correspond to: U=uninfected, S=Scott, O=Oka. A) β-actin tracing from figure 11a, panel B blot B) Figure 11a, panel B autoradiogram scan of the gene 14 specific transcripts.



B

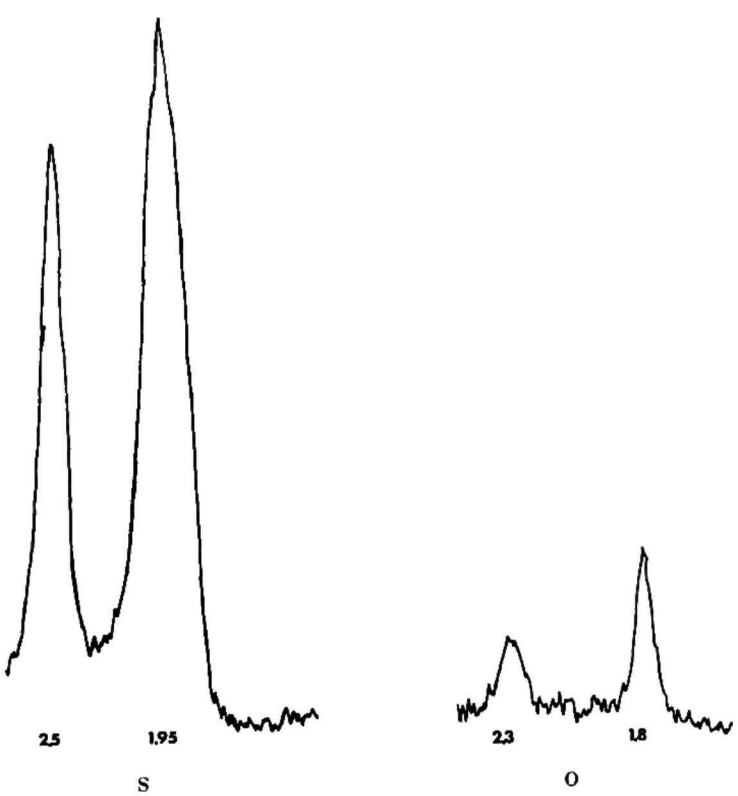


Table 1. Summary table of relative transcript abundance[a] normalized to β-actin[b]

	U	S	O	ratio S/O	normalize to β-actin
β-actin	63.2	43.4	106.0	0.4	1
SII/SpI:					
2.5kb(S)	-	117.6	-		
2.3kb(O)	-	-	20.5	5.7	14.3
1.95kb(S)	-	210.9	-		
1.8kb(O)	-	-	20.1	10.5	26.3
Eco Q:					
2.5kb	-	8.7	21.9	.4	1
1.5kb	-	11.2	53.2	.2	.5
Eco M':					
1.1kb	-	35.7	84.8	.4	1
3.4kb	-	102.0	221.0	.45	1

Roman numerals (I-IV) on the left designate different probes and various transcripts detected.

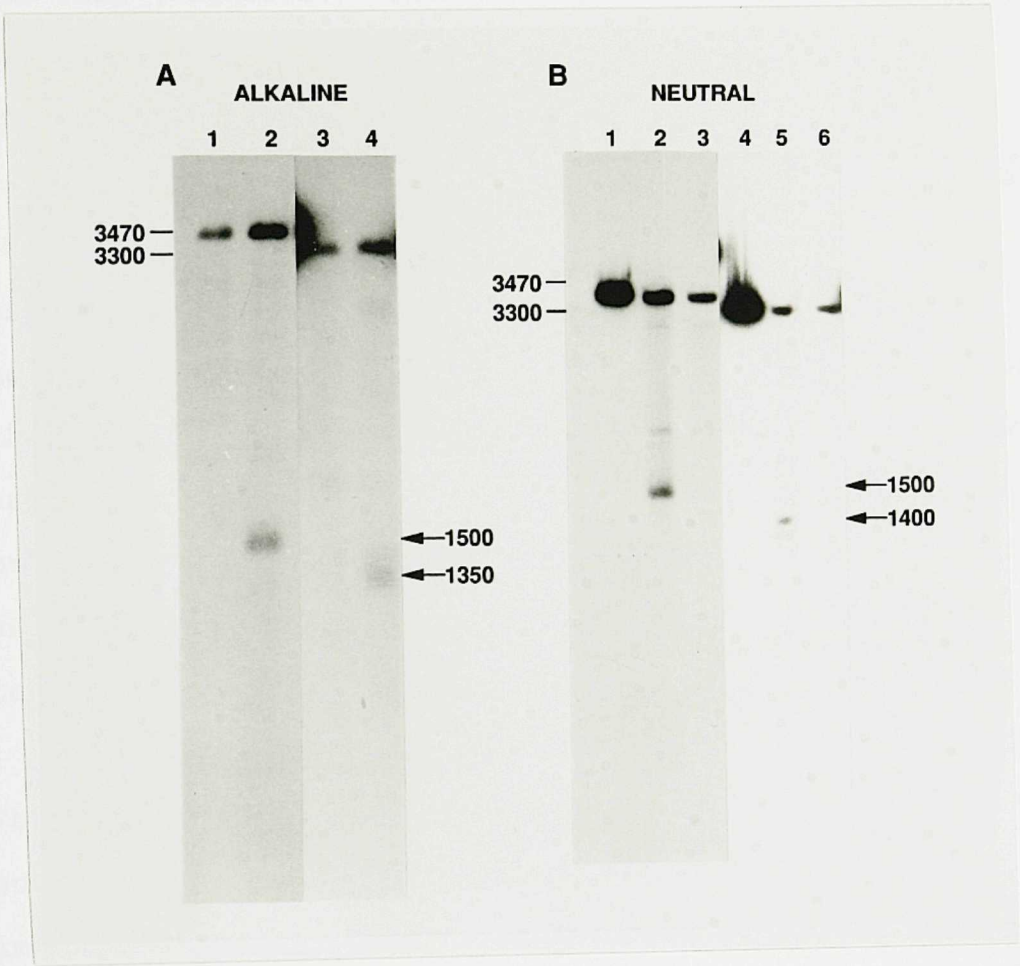
[a]The headings at the top represent uninfected (U), Scott (S), and Oka (O) relative RNA abundance as calculated by the area of the peaks from densitometry readings.

[b]The last two columns compare ratios of Scott to Oka RNA abundance for each transcript, with the ratios normalized to β-actin abundance.

S1 nuclease analysis of the 5' terminus of gene 14 encoding mRNA: The above data indicated that the gpV gene from VZV Scott was transcribed into 2.5kb and 1.95kb RNA species; in order to locate the likely promoter region for this gene, the 5' termini of these transcripts were mapped. Previous analysis of the DNA sequence in the ORF14 region indicated two potential in-frame ATG start sites for the open reading frame separated by 93bp, and numerous potential TATA-like sequences further upstream (Kinchington et al., 1986). Two probes were used as shown in figure 9, and the protection products were halved and run under neutral and alkaline conditions (see materials and methods). The first probe, a BstE II/BamH I fragment (see figure 9), yielded protected products of 1500bp for strain Scott under both alkaline (figure 13a, lane 2) and neutral conditions (figure 13b, lane 2). The same probe, generated from Oka DNA and hybridized with Oka RNA, yielded protected products of 1350bp for strain Oka under alkaline conditions (figure 13a, lane 4) and 1400bp under neutral conditions (figure 13b, lane 5). It should be noted that 50 to 100-fold more Oka RNA was required in experiments to visualize products generated from S1-digested hybrids. The sizes of the protected fragments are consistent with the presence of a 168bp deletion in strain Oka ORF14. These results also confirm the northern blot analyses, and show that the 2.5/1.95kb and 2.3/1.8kb transcripts are 5' co-terminal non-spliced RNA species. To obtain a precise assessment of the true 5' terminus, we used a small Acc I/Nde

FIGURE 13

S1 nuclease analysis of the 5' end of gene 14 transcripts. A BstE II/BamH I probe labeled at the 5'end (see fig. 9) was hybridized to VZV RNA or uninfected cell RNA and digested with S1 nuclease. The protected fragments were run on either alkaline (A) or neutral (B) gels. (A) lanes 1 and 3 are BstE II/BamH I probes from Scott and Oka DNA respectively, hybridized with uninfected cell RNA and digested with S1 nuclease. Lanes 2 and 4 show BstE II/BamH I probes from Scott and Oka respectively, hybridized with their respective RNAs and digested with S1 nuclease. (B) Lanes 2-3 and 5-6 are the other half of reactions from (A), lanes 1-2 and 3-4 respectively, run under neutral conditions. Lanes 1 and 4 are the Scott and Oka S1 labeled probes with no manipulations. Numbers on the side of the gels are sizes in bases.


A
ALKALINE
1 2 3 4
3470
3300
1500
1350
B
NEUTRAL
1 2 3 4 5 6
3470
3300
1500
1400

I probe (figure 9) in further S1 protection experments on Scott RNA, and analyzed the products on an 8% polyacrylamide sequencing gel. The protected fragments were approximately 80 to 83bp (figure 14) with major bands at 81bp and 82bp, placing the 5' transcription initiation site 36 to 37bp upstream from the second potential initiation codon. To confirm these data, we used the primer extension technique on both Scott and Oka RNA as described below.

Primer extension analysis of 5' termini of the VZV gene 14 mRNA: Primer extension analysis was performed on both Scott and Oka RNA using a primer specific for the gpV transcript (see materials and methods). When this primer was hybridized to Scott RNA and extended with reverse transcriptase, extension products of 106 to 109bp were detected with major bands appearing at 107 and 108bp (figure 15, lane 5). Strain Oka extension products, a single band at 107bp, were barely detectable and reflected amounts of RNA seen in the northern blot analysis (figure 15, lane 4). Primer extension products were then run in parallel with a dideoxy sequencing ladder generated using the gene 14 primer on an EcoR I P DNA template, to determine the site of mRNA initiation (figure 16). Figure 16a shows the cap sites for gene 14 mRNA, with potential TATA and CCATT boxes highlighted. We have indicated two residues as potential transcriptional start sites but, given the evidence that most eukaryotic mRNAs initiate at a purine residue, we favor the A at -37bp as the more likely site.

FIGURE 14

S1 nuclease analysis of the 5' end of Scott gene 14 RNA. A 5' end-labeled Acc I/ Nde I fragment (fig. 9), was hybridized to Scott RNA and digested with S1 nuclease. Protected products were run on an 8% denaturing polyacrylamide gel (lane A). Nothing was observed when uninfected cell RNA was used (data not shown). Lane M shows a DNA ladder from pUC 18 DNA digested and labeled with T4 polynucleotide kinase and ^{32}P-ATP. Numbers on the side of the gel are sizes in bases.

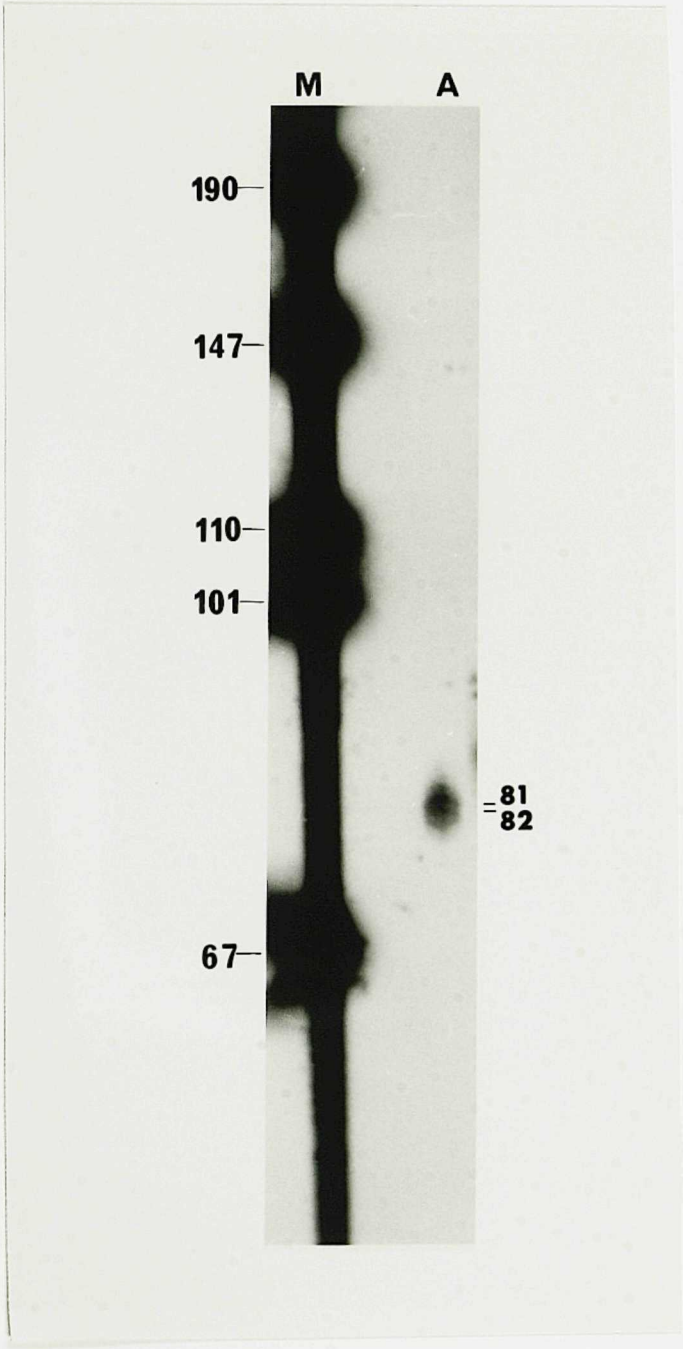

M
A
190
147
110
101
67
81
82

FIGURE 15

Primer extension analysis of the 5' end of gene 14 RNA. A gene 14-specific primer (lanes 4-6) or gene 67 primer (lanes 1-3) was hybridized with VZV or uninfected cell RNA. Lanes 2 and 5 are reaction products using Scott infected cell RNA and lanes 1 and 4 are reactions using Oka infected cell RNA. Extension of these primers with reverse transcriptase yielded cDNA fragments that were fractionated on an 8% denaturing polyacrylamide gel. Lanes 7 and 8 are molecular weight standards with sizes given in bases. No extension products were observed with uninfected cell RNA (lanes 3 and 6). The autoradiogram was overexposed to detect the band in lane 4.

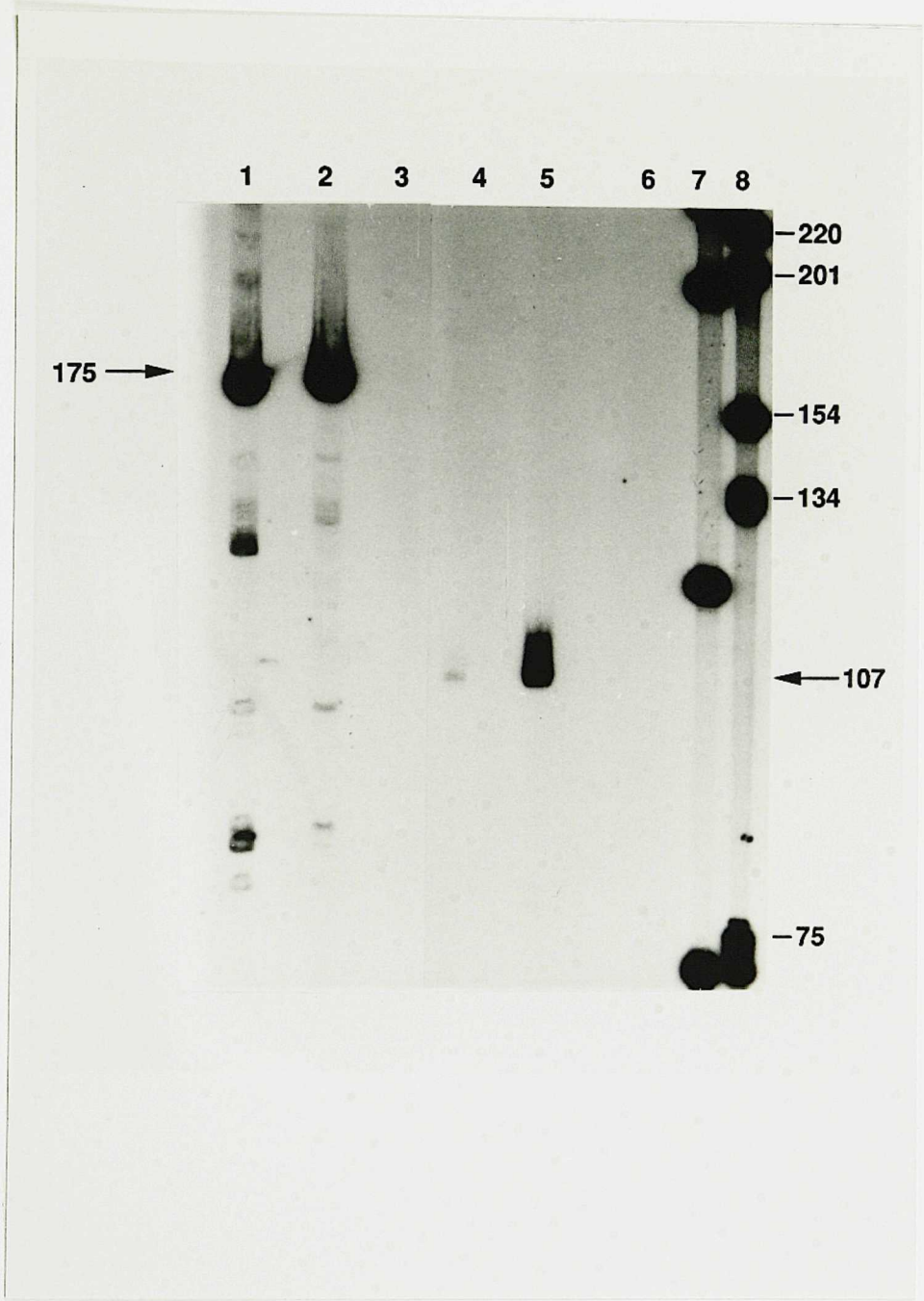
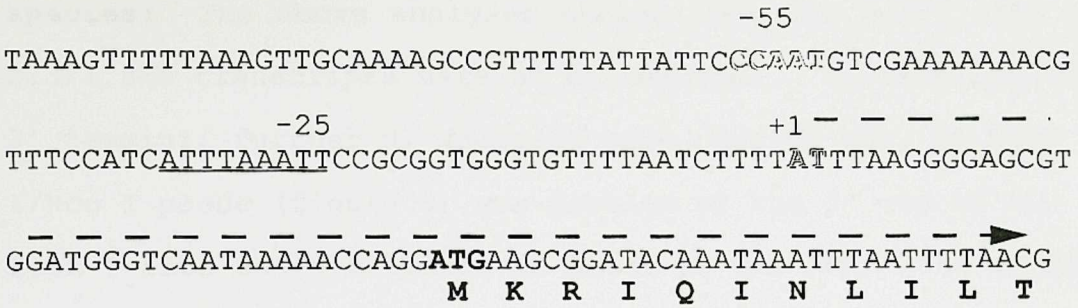

1
2
3
4
5
6
7
8
220
201
175
154
134
107
75

FIGURE 16

Determination of the gene 14 cap site and the nucleotide sequence of the 5' promoter region. (A) Nucleotide sequence of the 5' transcription terminus. The site of transcription initiation is +1. Potential TATAA and CCAAT boxes are underlined and outlined at -25 and -55bp respectively. Bold letters under the sequence show amino acids that code for the N-terminus of gpV. (B) Primer extension products made similar to those shown in fig. 15 were run in parallel with a sequencing ladder on denaturing 8% polyacrylamide gels. The sequencing ladder was generated by using a plasmid (sp1) containing the Scott Eco R1 P fragment with the gene 14 specific oligonucleotide. Since the primer sequence is antisense to the mRNA, the generated sequence ladder is also antisense to to the mRNA. Thus, the cap site is computated by reading the complementary base pairs from this ladder. Numbers on the left of B are sizes in bases.

A


-55
TAAAGTTTTTAAAGTTGCAAAAGCCGTTTTTATTATTCCCAATGTCGAAAAAAACG
-25
+1
TTTCCATCATTTAAATTCCGCGGTGGGTGTTTTAATCTTTTATTTAAGGGGAGCGT
GGATGGGTCAATAAAAACCAGGATGAAGCGGATACAAATAAATTTAATTTTAACG
M K R I Q I N L I L T

B


T G C A
108
107
C
T
T
T
T
A
T
T
T
A
A
5'END

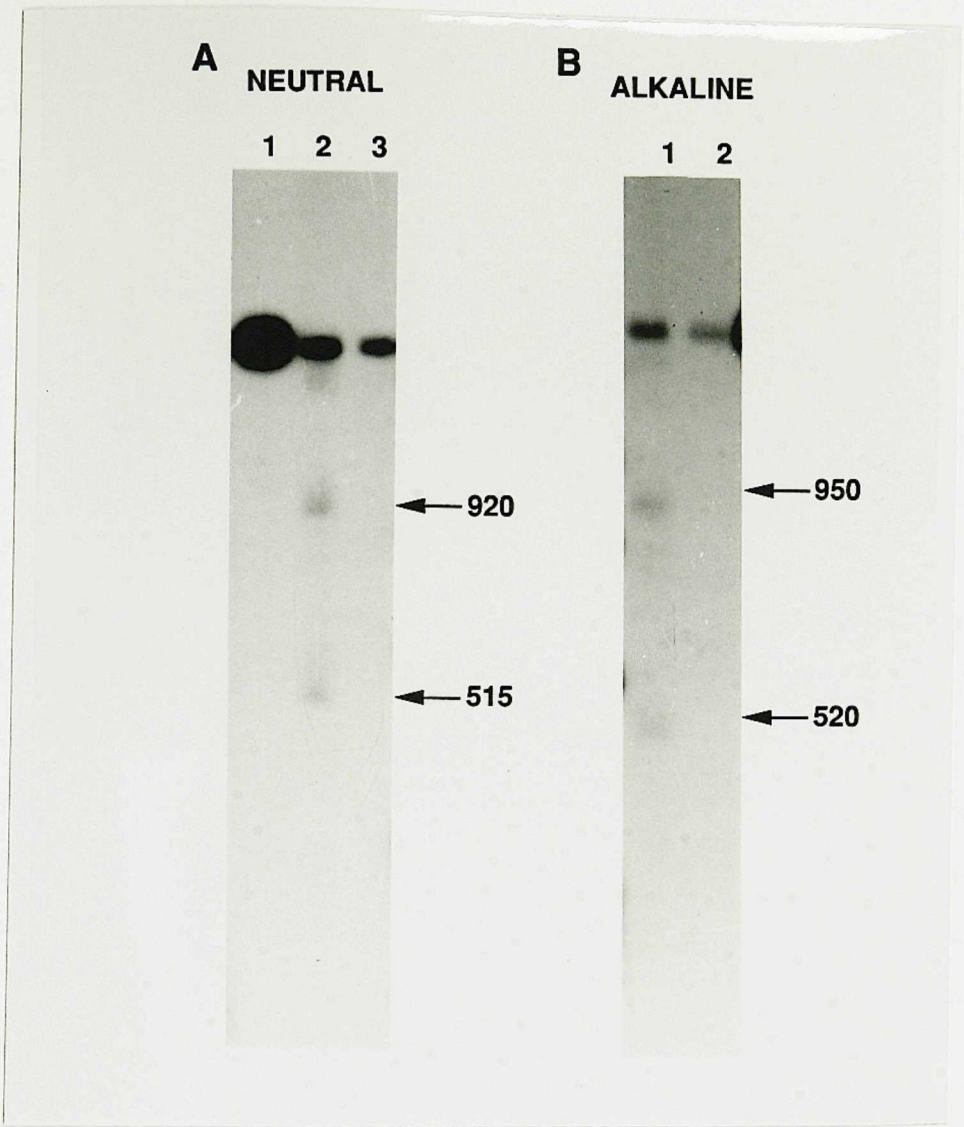
S1 nuclease analysis of 3' terminus of gpV mRNA species: The above analyses showed that the 2.5/1.95kb and 2.3/1.8kb transcripts were 5' co-terminal. To localize the 3' termini, further S1 analyses were carried out. An EcoR I/Nco I probe (figure 9) was labeled at the 3' end of the EcoR I site and hybridized to Scott or Oka RNA, with similar results for each. After S1 digestion, the products were subjected to neutral and alkaline gel electrophoresis. Under both conditions, fragments of about 950bp and 520bp were observed (figure 17a and b), supporting the earlier observations that gene 14 transcripts have different 3'termini, about 500bp apart, and implying a lack of splicing. The 2.5/2.3kb and 1.95/1.8kb termini map 10-20bp downstream from ATAAA and ACGTAAA sequences, respectively (figure 17c); these sequences deviate somewhat from other eukaryotic poly(A) sites but there are precedents for variation on the general theme (Davison et al., 1986b; Frink et al., 1983, Dowhower et al., 1981; Nevins, 1983). GU-rich sequences analogous to those recently described in HSV (Mclaughlin et al., 1985) are also observed 24-30bp downstream of the poly(A) site and are shown in figure 17c.

Transcript analysis of gpIV in VZV Scott

Northern blot analysis of VZV gene 67 RNA: Previous northern hybridization studies mapped 3 polyadenylated transcripts (3.6, 2.8 and 1.65kb) to the gpIV ORF (Ostrove et al., 1986; Kato et al., 1989). Reinhold et al. (1988)

FIGURE 17

S1 nuclease analysis of the 3' ends of VZV gene 14 RNA. 3'end-labeled EcoR I/Nco I fragments (see fig. 9) were hybridized with VZV RNA and digested with S1 nuclease. The protected products were run on neutral (A, lane 2) and alkaline (B, lane 1) gels. Uninfected RNA hybridized with these same fragments (A, lane 3 and B, lane 2) or probe fragments untreated (A, lane 1), and digested with S1 nuclease detected no protected fragments (C) Nucleotide sequence of the 3' termini of gene 14 RNA. The 3' termini are indicated by asterisks. Potential polyadenylation signals are underlined, while GU-rich regulatory signals are in bold letters. Numbers on the sides of the gels are sizes in bases.



C

1.95kb and 1.8kb RNA

```
                                    ****
1   TCCGTTGCTGTTCATAAACAGAAACCAACCAAACGCGTCTGTGTATATCATTTTATTACA
61  TTCGCAACACATCTACTGTCTTGACAACATTTAAAAATCCATTAAAGAGCCATTTTCCAT
121 TTTTAGGGGGGGGTGTGGATTATATCCATCAAGCTGAAAATCGTCCCATTTAAAGTCGTT
181 TATATCTGTTACATTTCGAATAATTTTAAGGCAAGGAAAAGGTTTTGGGGATCGAGCTAG
241 CTGCACTTTTAAAGCATCTATATGCTTCAAGTAAATATGTGCATCCCCCATTGTATGAAT
301 TAAATCTCCGGTTTTAAGTCCTGTAACATGCGCTACTATGTAGGTAGAAGTGCATATCCA
361 GCAATGTTGAACGGTACCCCAAGGCCCATATCCCCCGATCTCTGGTATACTTGGCAGGAT
421 AATTCACCGTTTGCAACGTAAAACTGACATAACGTGTGACATGGTGGTAGTACCATTAAG
                    ****
481 GGGATATCCTTTGGATTCC       2.5kb and 2.3kb RNA
```

extended this work with single stranded RNA probes and detected two RNAs of 3.6kb and 1.65kb that were transcribed from left to right (toward the TR_S) in the prototype VZV genome arrangement. In order to confirm and extend these results we used two probes, a DNA fragment containing gpIV ORF sequences and an oligonucleotide specific for the gpIV ORF, in northern hybridization experiments. An AccI fragment (probe A, figure 18), when hybridized to poly(A)$^+$ or poly(A)$^-$ VZV RNA, recognized only transcripts in the poly(A)$^+$ tracks and was specific for infected cells (figure 19). We estimate the transcripts to be 3.6kb, 2.7kb and 1.65kb in size. An oligonucleotide probe that hybridizes to a 20bp sequence within the gpIV ORF (probe B, figure 18) detected the same transcripts as the AccI probe, and indicated that all three are transcribed from left to right (towards the TRs) in the prototypic arrangement of the VZV genome (figure 19). Further analysis with a probe downstream from the gpIV ORF detected the 3.6kb transcript plus a novel transcript of 2.15kb (data not shown). Our conclusions, based on the above data and that of others, are that the 1.65kb transcript codes for gpIV specifically, that the 2.7kb transcript is a readthrough from gene 66 that terminates close to the 1.65kb transcript terminus, and that the 3.6kb transcript initiates at or near the 1.65kb 5' terminus, but terminates downstream of the gpI ORF68. Finally, both Scott and Oka strains appeared to have identical transcript patterns for this

FIGURE 18

Summary diagram of probes used for transcription mapping of gene 67. The diagram shows the various DNA fragments used for both northern hybridization (probes A and B) and S1 nuclease analysis (probes C and D). The line above represents a portion of the U_S region of the genome, with the nucleotide numbers above the line and restriction sites (Ba; BamHI, A; AccI, D; DdeI, B; BstEII) below. Shown on the bottom are the locations of the flanking open reading frames to gene 67.

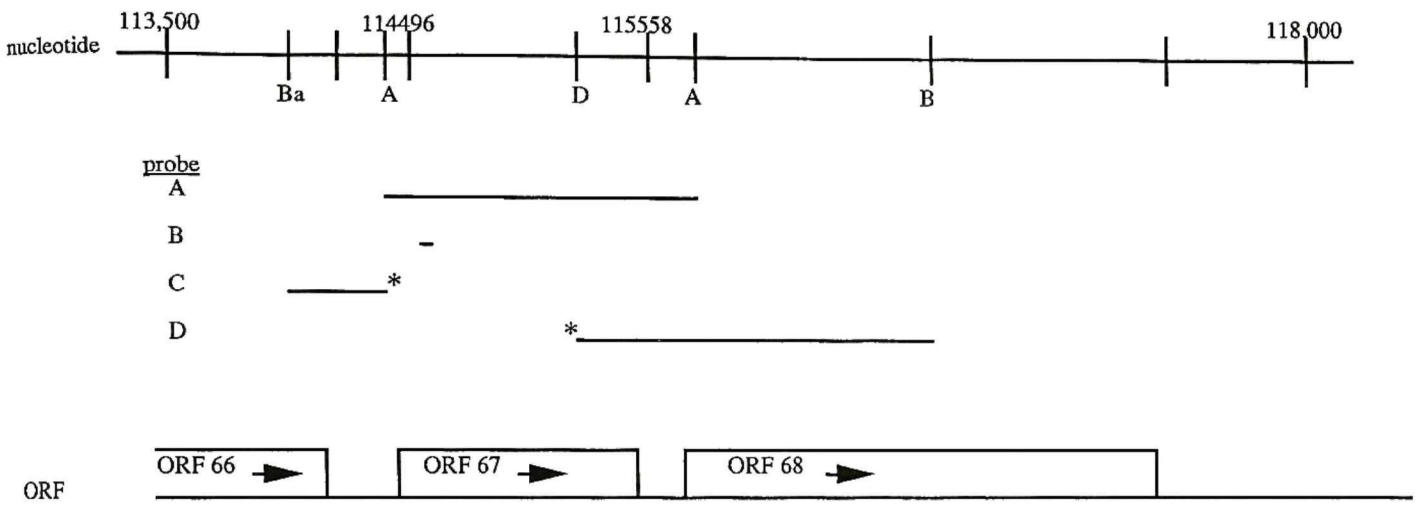
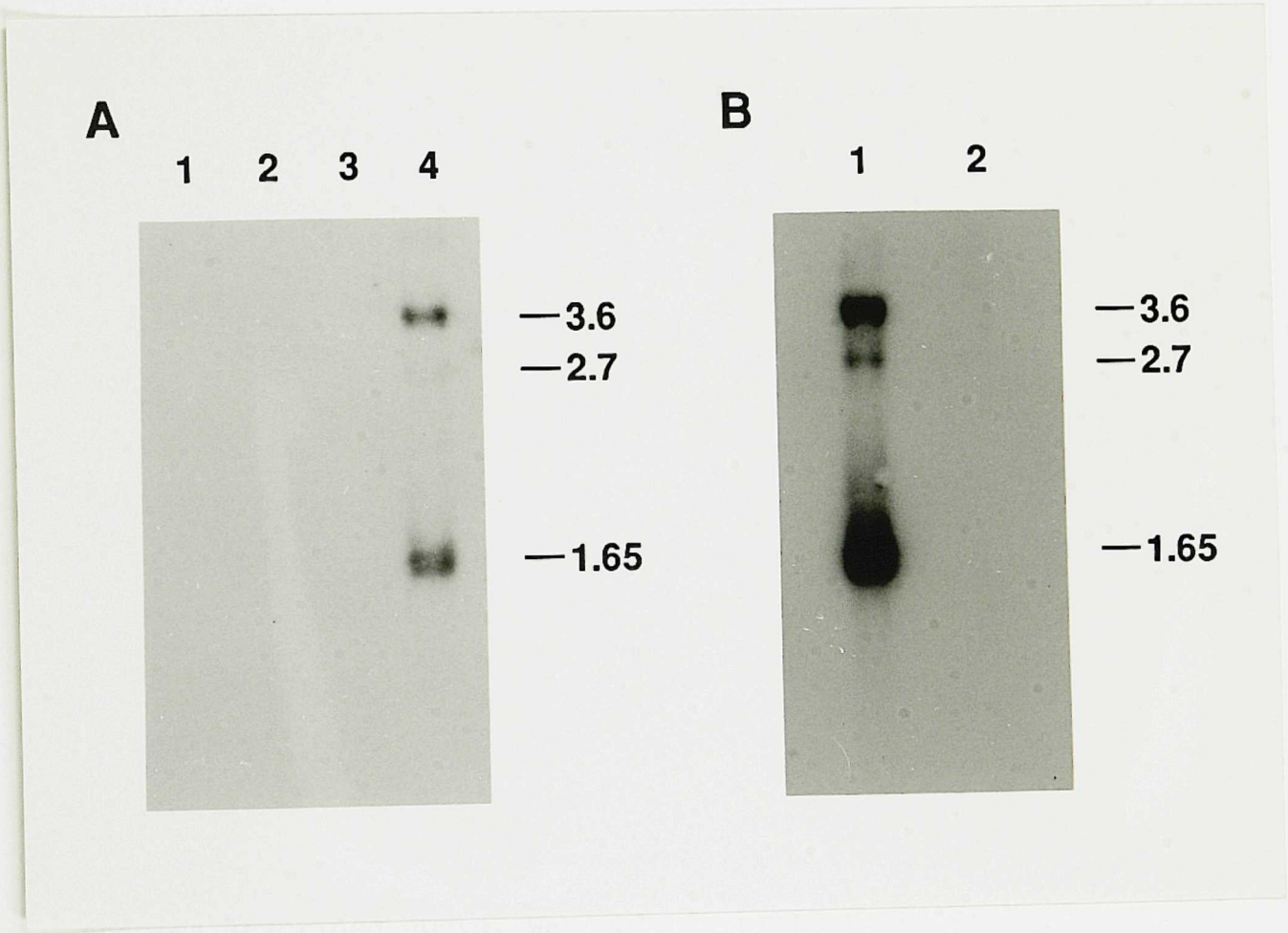

nucleotide
113,500
114496
115558
118,000
Ba
A
D
A
B
probe
A
B
C
D
*
*
ORF
ORF 66
ORF 67
ORF 68

FIGURE 19

Northern blot analysis of gene 67. A) A northern hybridization experiment comparing poly(A)$^+$ RNA to poly(A)$^-$ RNA from uninfected and VZV-infected cells. Lane 1 is uninfected cell poly(A)$^-$ RNA, lane 2 is uninfected cell poly(A)$^+$ RNA, lane 3 is VZV-infected cell poly(A)$^-$ RNA, and lane 4 is VZV-infected cell poly(A)$^+$ RNA. The blot was probed with an Acc I DNA fragment (probe B, fig. 18). B) This is a northern blot showing RNA probed with an oligonucleotide OPL5 (probe A, fig. 18). Lane 2 is uninfected cell RNA, and lane 1 is VZV-infected cell RNA. Numbers on the side of the gels are sizes in kilobases.


A
1
2
3
4
—3.6
—2.7
—1.65
B
1
2
—3.6
—2.7
—1.65

region of the genome as illustrated by a northern blot comparing Scott and Oka using a Bam K probe (figure 20).

S1 nuclease analysis of the 5' end of the gene 67 transcript: In order to map the 5' terminus of the major 1.65kb transcript encoding gpIV, we performed S1 nuclease protection assays. Probe C (figure 18) was hybridized to VZV-infected cell RNA and digested with various concentrations of S1 nuclease. Two major protection products migrating at 63bp and 64bp are observed (lanes 1-3, figure 21) and indicate a 5' terminus at 114405bp, 90bp to 91bp upstream of the proposed translational initiation codon at 114496bp. No protection products are seen when this probe is hybridized and digested with uninfected cell RNA (figure 21, lanes 4-6). Bands appearing at the top of lanes 1-6 migrating at 426bp are either the result of probe to probe reannealing or the presence of a transcript substantially larger than 1.65kb. In lanes 1-3 (infected cell RNA), the amount of this larger band is much greater than that observed with probe and uninfected RNA (lanes 3-6), indicating that a transcript, most likely the 2.7kb species described above, was able to protect the entire Acc I/BamH I fragment (figure 18), in agreement with our conclusions from the previous section.

Primer extension analysis of the 5' end of gene 67 RNA and determination of the transcription initiation site: We hybridized an oligonucleotide (OPL5, see materials and methods), with VZV RNA and synthesized a cDNA product using reverse transcriptase. A product of 162 to 163bp was observed,

FIGURE 20

Northern blot analysis of VZV poly(A)$^+$RNA probed with a Bam H I K fragment. Lane 1 is uninfected cell RNA, lane 2 is Scott-infected cell RNA, and lane 3 is Oka-infected cell RNA. The RNA was from a poly(A)$^+$ fraction used in previous mapping studies for gpV. Numbers on the right are sizes of transcripts in kilobases.

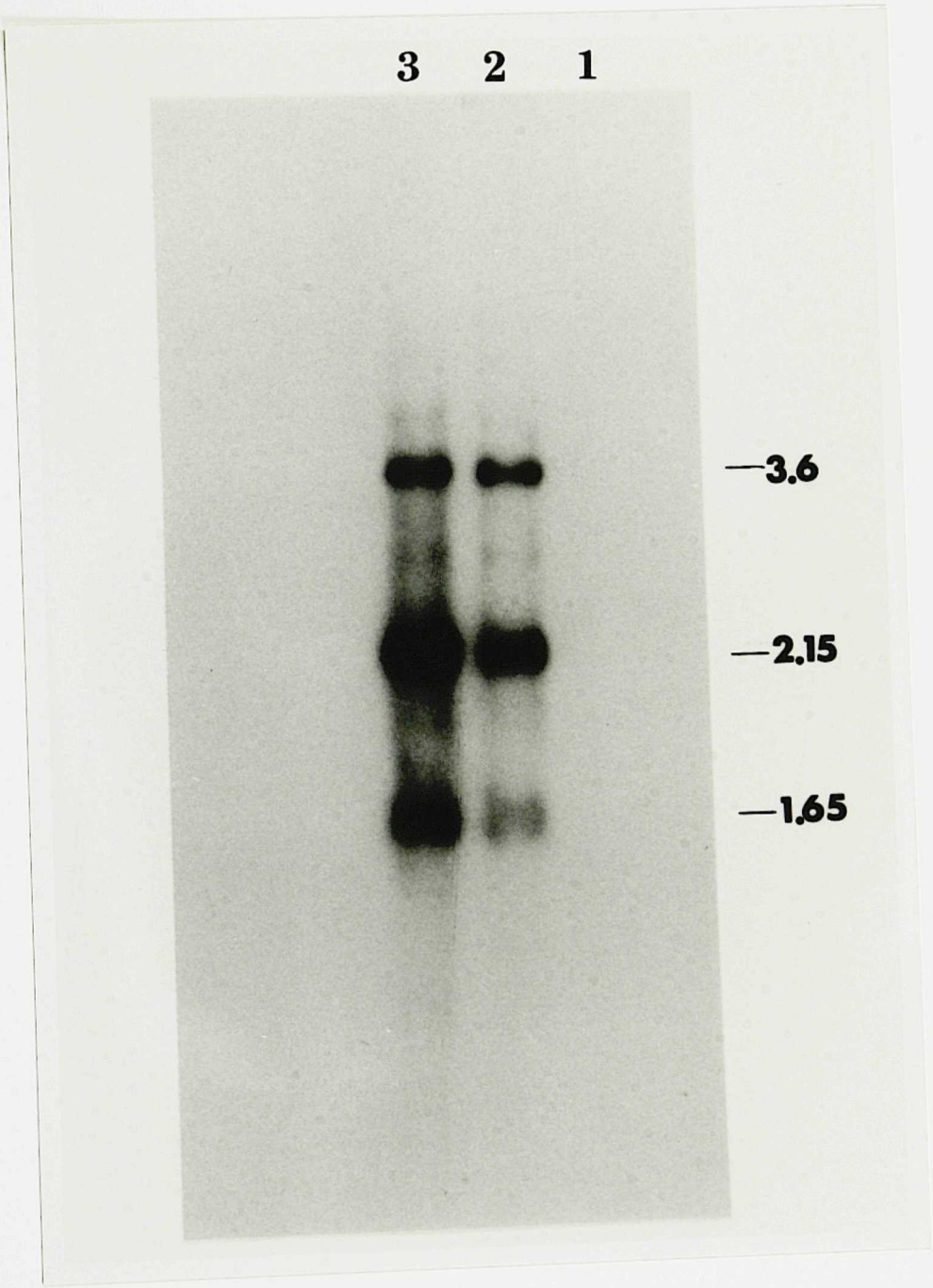
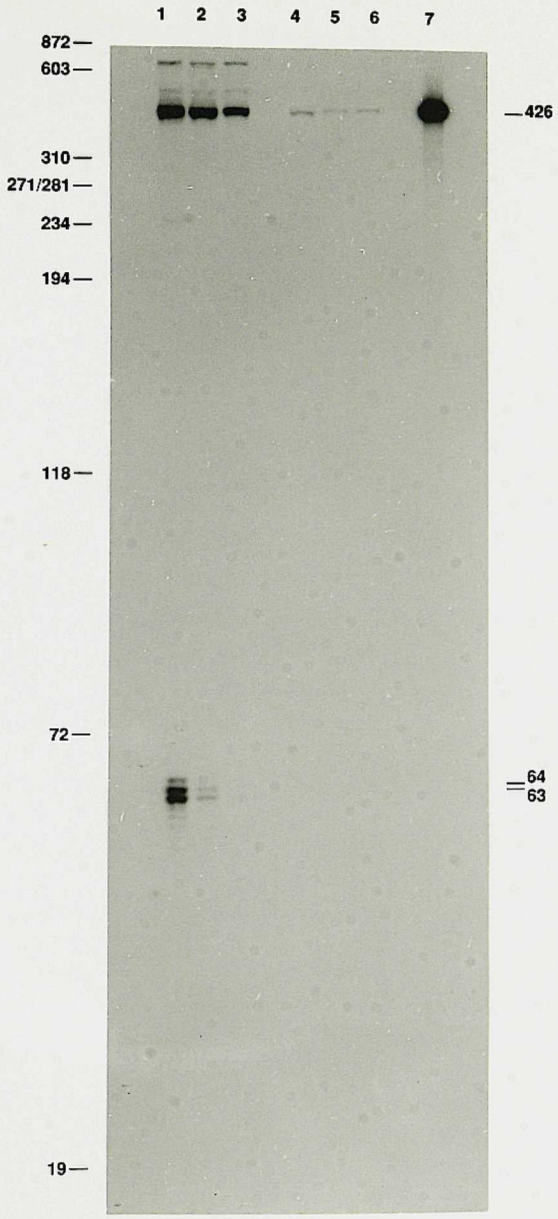

3
2
1
—3.6
—2.15
—1.65

FIGURE 21

S1 nuclease analysis of the 5' end of gene 67 RNA. An Acc I/BamH I probe (see figure 18, probe C) was hybridized with VZV-infected cell RNA (lanes 1-3), or uninfected cell RNA (lanes 4-6) and digested with S1 nuclease. Products were run on an 8% denaturing polyacrylamide sequencing gel. Various concentrations of S1 nuclease were used; 100 units lanes 1 and 4, 10 units; lanes 2 and 5, and 1 unit; lanes 3 and 6. Lane 7 is the probe untreated. Molecular size in bases is shown to the left for a molecular weight marker and to the right for S1 protected products.


1 2 3 4 5 6 7
872
603
310
271/281
234
194
118
72
19
426
64
63

which confirms the S1 analysis (figure 22b). This primer extension product was then run in parallel with a sequencing ladder generated by using OPL5 as a primer on a BamH I K DNA template (see materials and methods). From this, the 5' terminus is most likely to be the G residue at position 114405, since most RNA polymerase II molecules transcripts initiate at a purine nucleotide. We do, however, see stuttering in both primer extension and S1 nuclease analyses. This has been observed with other herpesvirus transcripts (see gene 14 transcript analyses) and we cannot rule out the possibility that the T at 114404 is also an initiation site for this VZV mRNA (Frink et al., 1983). Upstream at -25bp from the capsite is an AT-rich sequence ATAAAA that may serve as the TATA box; further upstream at -85bp there is a TATAAA sequence, representing a more usual TATA motif, but its position makes it a less likely candidate (figure 22a). At -65bp from the capsite is a consensus CCAAT sequence (figure 22a).

S1 nuclease mapping of the 3' termini of the gene 67 transcript: To determine the 3' terminus of the 1.65kb RNA, we performed protection assays using probe D (figure 18) that was hybridized with VZV RNA and digested with S1 nuclease. Protected hybrids were analysed on denaturing polyacrylamide sequencing gels and protected bands migrating at 400bp were observed (figure 23a, lanes 1 and 2). No bands are seen when probes were hybridized and digested with uninfected cell RNA (figure 23a, lanes 3 and 4). This would place the 1.65kb RNA 3' terminus around 23bp downstream (115617bp) from ATTAAA or AATAAA

FIGURE 22

Primer extension analysis of the 5' end of gene 67 RNA. The OPL5 oligonucleotide was hybridized with VZV-infected cell RNA, and extended with reverse transcriptase in the presence of dNTPs. The extension product was then resolved on a denaturing polyacrylamide sequencing gel. The OPL5 oligonucleotide was also used as a primer for sequencing on a plasmid containing the BamH I K DNA insert. The sequencing products were run in parallel with the primer extension fragments. A) Above the autoradiogram is shown diagramatically the start site of the gene 67 RNA in relation to its surrounding sequence. The initiating ATG codon is in bold outline, the TATA box is underlined, the CAT box is in bold print, and the cap site is labeled above as +1. The dashed line above the sequence represents the mRNA. Amino acid designations for the N-terminus of the protein are listed below the sequence. B) Lanes 1 and 2 are two different primer extension reactions: uninfected RNA in lane 1; infected cell RNA in lane 2. The lanes designated with A,C,G,T, are lanes terminating with those nucleotides. On the left of B are sizes of extension products in bases.

A


-65
TTTGAAATACTAATATAAATAACATAACCAATAGAAACTTAATACAGAG
-25
+1
TCACGCCCCATTACAACAAGGATAAAA CACGGGATCATTTTCTTAACAT
TGTAGTAGCGCTGAAAAGCGTCCCCTCCCCCGGCTCACAGAGCTGCTCTTC
GGTGTAGTTGGGTATACTGGTGCGCCTCATTTAATCGCGATGTTTTTTAATC
M F L I
CAATGTTTGATATCGGCCGTT
Q C L I S S V

B

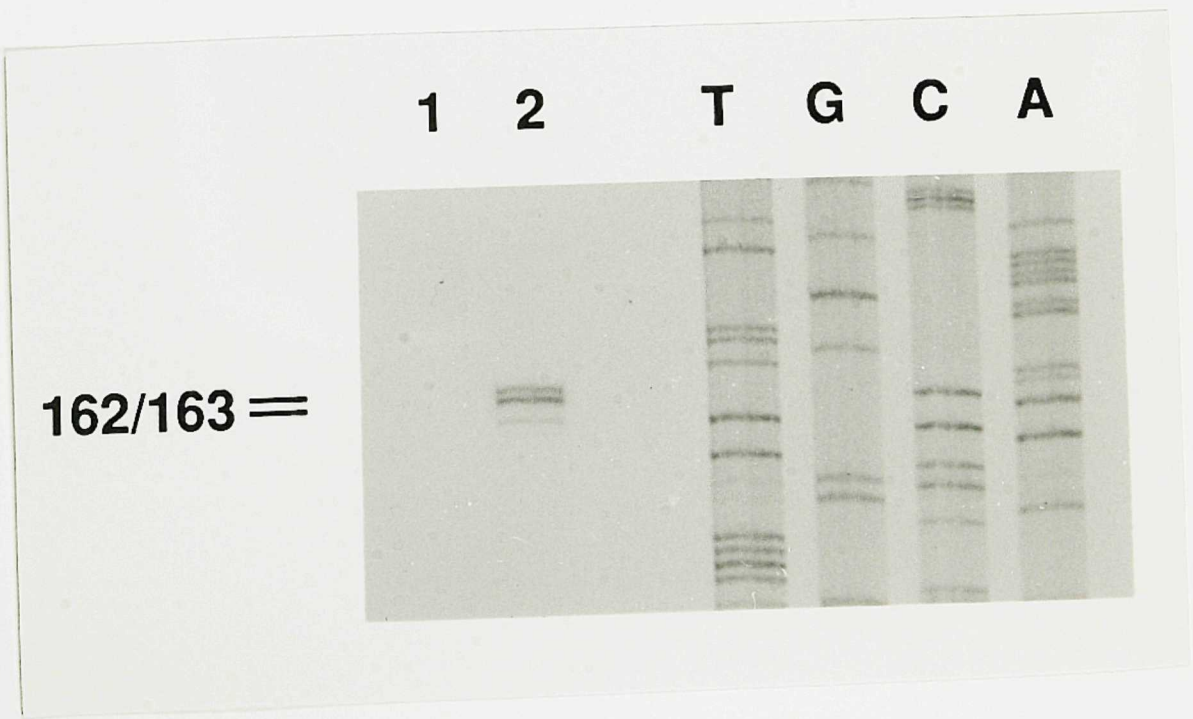
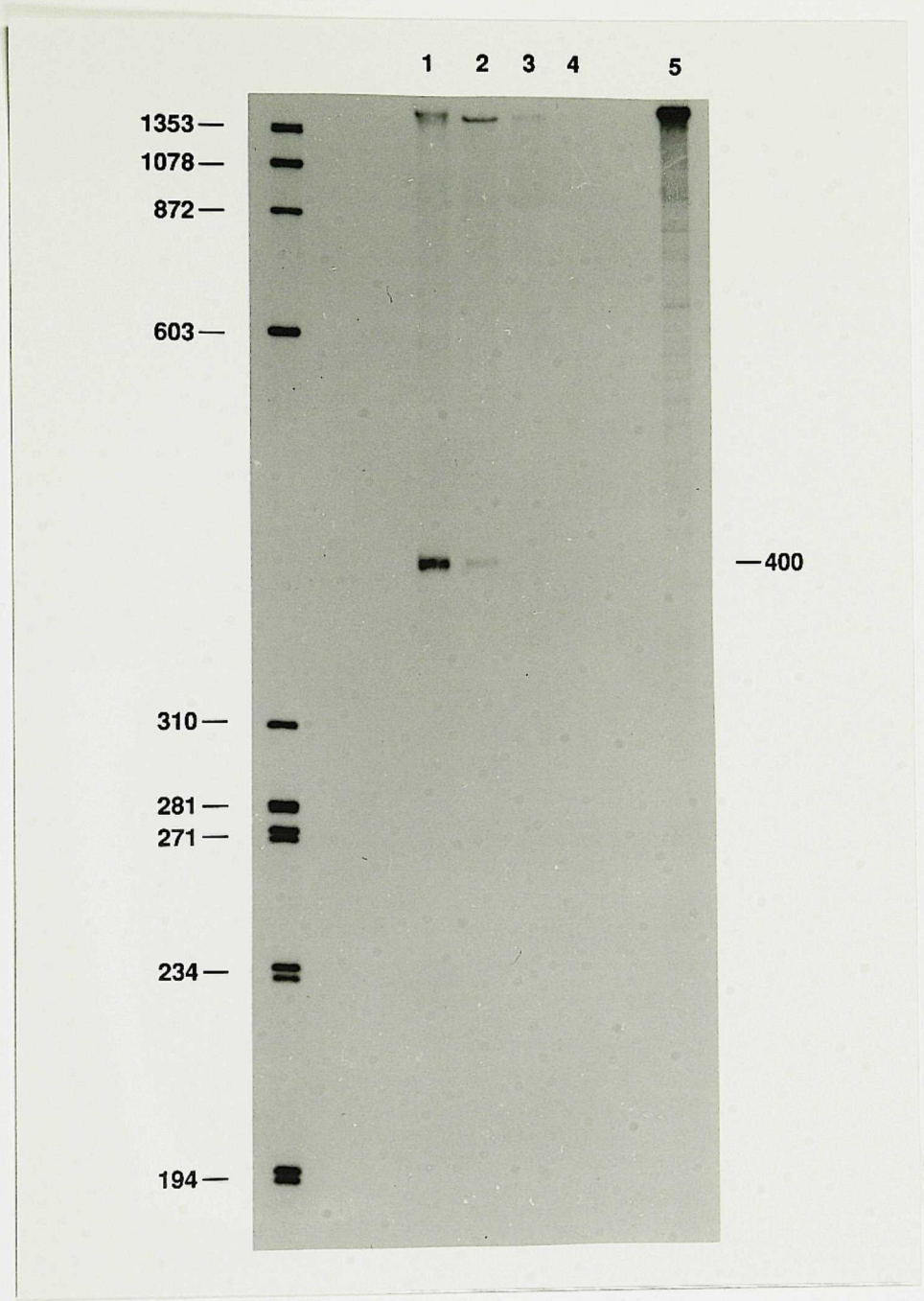

1 2
T G C A
162/163 =

FIGURE 23

S1 nuclease digestion of the 3' end of the gene 67 transcript. A) A Dde I/BstE II fragment (probe D, fig. 18) was hybridized with VZV-infected cell RNA (lanes 1 and 2) and uninfected cell RNA (lanes 3 and 4). The hybridization reactions were digested with 10 units (lanes 1 and 3) and 1 unit (lanes 2 and 4) of S1 nuclease. S1 digestion products were resolved on 4% denaturing polyacrylamide gels. MM is a molecular weight marker with sizes in bases shown on the left and on the right the S1 protection products are shown in bases. B) Shows the sequence near the 3' end of the 1.65kb transcript. Bold letters are the poly(A) sites, the asterisks above the second line of sequence is the 3' end of the 1.65Kb transcript, and a GU-rich motif is underlined.

A



B

TTTGTTAAATTAGAACTAATTATCCCGGATTTTAT**ATTAAATAAA**CTATATGCGTTT

****

TATTTAGCGTTTTGATTACGCGTTGTGATATGAGGGGAAGGATTAAGAATCTCCTAA

CTATAAGTTAA

consensus polyadenylation signals. In addition, a GU-rich sequence downstream of the poly(A) signal similar to that predicted by Mclaughlin et al. (1985) has been outlined in figure 23b. The combined data from S1 and primer extension analyses indicate that the body of the major gpIV transcript is 1212bp, consistent with a polyadenylated transcript of 1.65kb detected from northern blots. This suggests a polyadylation length of about 400bp, somewhat larger than the normal 200-250bp tails found in other eukaryotic messages. There is no indication of transcript splicing in the gpIV region of VZV DNA; RNA splicing for homologous genes in HSV-1 U_S has not been found (Rixon and McGeoch, 1985).

Once again, as noted in the 5' S1 analysis, the proportion of probe protection in infected cell lanes compared to uninfected cell lanes is much greater. The likely explanation is that a long transcript, probably the 3.6kb RNA, co-5'terminal with the 1.65kb RNA, hybridizes to the whole probe, consistent with our earlier conclusions.

Functional studies on potential gpIV and gpV regulatory sequences

One major question arising from the above analyses was why VZV strain Oka synthesized barely detectable amounts of gene 14 transcripts, while other genes, including gene 67 appeared to be transcribed at normal levels. The regulation of gene expression can occur on many levels, and most commonly, hinges on the rate of RNA initiation. However, it should be

noted that post-transcriptional modifications affecting mRNA stability, transport, splicing, or translational efficiency are all well documented mechanisms for gene regulation (reviewed in Mitchell and Tjian, 1989; Roizman and Sears, 1990). In the case of gpV gene expression, lower levels of gpV protein synthesized in infected cells correlate very well with observed amounts of RNA transcripts (see gpV protein analysis below). This suggests that the Oka defect is probably due to a faulty transcriptional mechanism. We decided to test this possibility by cloning sequences of interest into reporter gene vectors and testing them in transient assays.

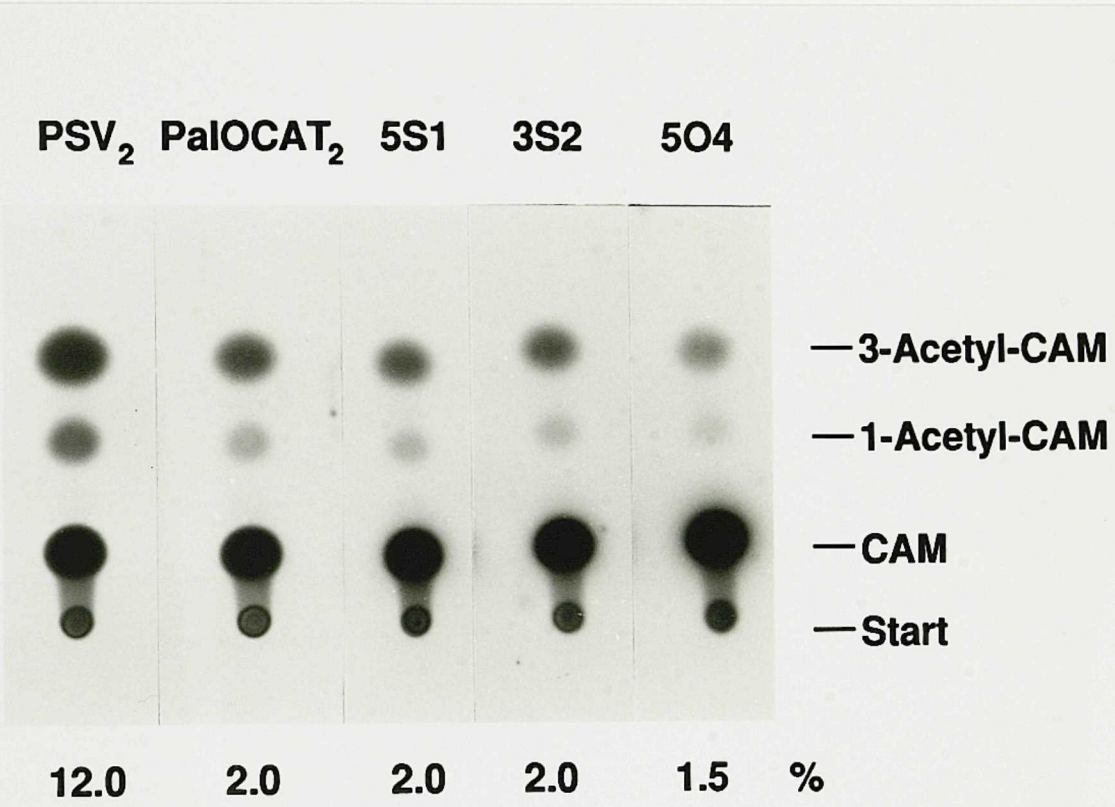
Determination of the 42bp repeating sequences for enhancer activity in transient expression assays: The data presented above show that Oka is markedly deficient in transcription from gene 14, compared to another VZV strain. Examination of the sequences of the structural genes and the potential promoter regions (+1 to +150bp) for Oka and Scott reveals no substantial differences (data not shown), apart from the numbers of copies of the R2 repeat sequence. Many such repetitive DNA sequences have been characterized as transcriptional enhancers in a variety of systems, including herpesviruses (Reviewed in Roizman and Sears, 1990; Mitchell and Tjian, 1989), and the number of repeats present are often directly related to the strength of the enhancer (Mitchell and Tjian, 1989). We tested the ability of the R2 sequences from both strains Scott and Oka to act as enhancers, cloning

them into the conventional test plasmid, $pA10CAT_2$ (see appendix). This vector contains a minimal promoter derived from SV40, cloned to drive expression of a chloramphenicol acetyltransferase gene (CAT). Insertion sites are available both 5' and 3' to the CAT gene. We chose to test two constructs for the Scott repeats (5' and 3' locations) and one for the Oka repeats (5' location) (see the appendix). Constructs were transfected into human fibroblast cells, permissive for VZV infection, which were harvested 3 days post VZV infection and assayed for CAT activity. Figure 24 shows that the R2 repeats were unable to increase the activity of an early SV40 promoter. Infection of transfected cells with VZV also had no effect (data not shown). pSV_2, which contains the SV40 72bp enhancers in addition to the SV40 early promoter ($pA10CAT_2$) showed a >5-fold increase in promoter activity over the $pA10CAT_2$ backround (figure 24). To exclude possible difficulties with the human cell line, additional (cv-1, vero, and LTK^-) cells were used but gave similar negative results (unpublished observations).

Determination of promoter activity from gene 14 upstream sequences: Determination of the 5' terminus for the gene 14 RNA transcripts allowed us to select likely promoter sequences for fusion with a reporter gene; we could then test the activity of this promoter in transient assays. (Sequencing analysis to -200bp of the Oka gpV promoter region revealed no differences between Scott and Oka. Since the 42bp repeats were unable to act as classical enhancers,

FIGURE 24

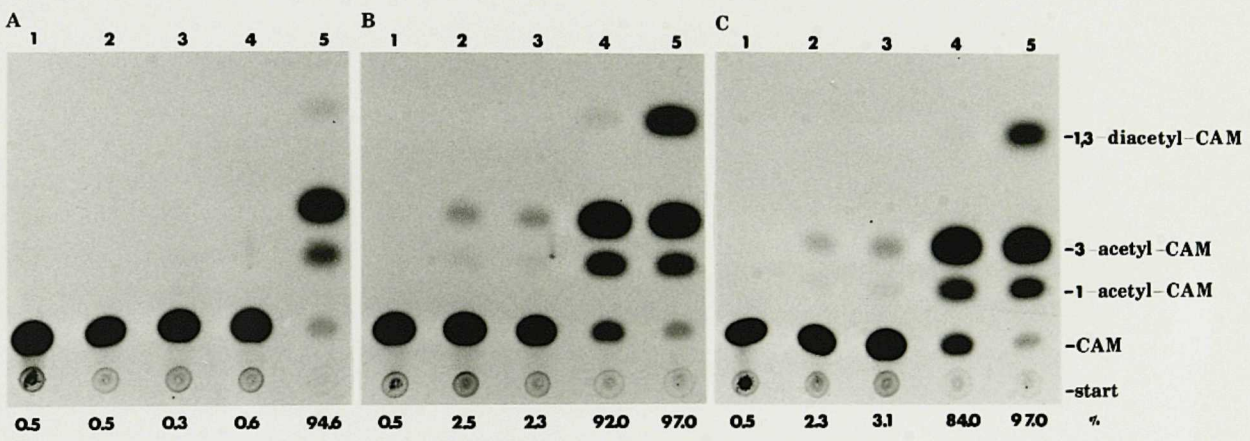
Examination of 42bp repeats for enhancer activity. SV40 promoter CAT plasmids containing VZV 42bp repeats were transfected into HFF cells. The cell extracts were then assayed at 48-72 hrs. post-transfection for CAT activity as described in the materials and methods. Unacetylated (CAM) and acetylated forms of chloramphenicol are shown. Lane 1, pSV_2; lane 2, $pA10CAT_2$; lane 3, 5S1; lane 4, 3S2; lane 5, 5O4. The numbers underneath the figure are percent conversion from CAM to the acetylated forms.


PSV2
PalOCAT2
5S1
3S2
5O4
—3-Acetyl-CAM
—1-Acetyl-CAM
—CAM
—Start
12.0
2.0
2.0
2.0
1.5
%

another possibility for the lack of gpV production in strain Oka, was that Oka was defective in some transacting factor.) Construction of the Scott and Oka gpV promoter/CAT fusions, S6 and O14, is described in the appendix. Both plasmid constructs were tested in transient assays with and without virus infection as shown in figure 25. Without virus infection, the promoters exhibited low levels of activity (figure 25a, lanes 2-3). Virus infection with Scott or Oka significantly increased activity of these promoters, but their activity was still below that of another promoter (from gene 67, see below) which we have tested (figure 25b, lanes 2-3; and figure 25c, lanes, 2-3). Control plasmids with no regulatory sequences (pCAT3M alone) had little activity in Scott and Oka infected cells (figure 25a,b,c; lane 1). In addition, we isolated clones that had the promoter in the opposite orientation and these also showed no activity in transient assays even when transactivated by viral infection (data not shown). These data show that we have defined a pivital region for ORF14, and that viral encoded or viral induced factors are required for detectable expression from it. Interestingly, no difference was observed between Scott or Oka virus used in super-infection. (There may be several reasons why we observe low activity from these promoters as well as the inability to detect a difference between the two strains in this assay, and they are described in the disscussion.)

FIGURE 25

Transactivation of the gpV promoter by VZV strain Scott and Oka. HFF cells were transfected with pCAT3M, S6 (Scott gpV promoter/CAT fusion), O14 (Oka gpV promoter fusion), or G4-3 (Scott gpIV promoter fusion). The cells were either left untreated or infected with VZV 24 hrs. post transfection. All cells were harvested 72 hrs. post-transfection and extracts were made and tested for CAT activity. Panel A, uninfected cells, Panel B, infected with VZV Scott and Panel C, Oka-infected cells. pCat3M, lane 1, all panels; S6, lane 2, all panels; O14, lane 3 all panels; G4-3, lane 4, all panels. A positive control pSV2 is in lane 5 in all panels. The numbers underneath the figure are percent conversion from CAM to the acetylated forms.

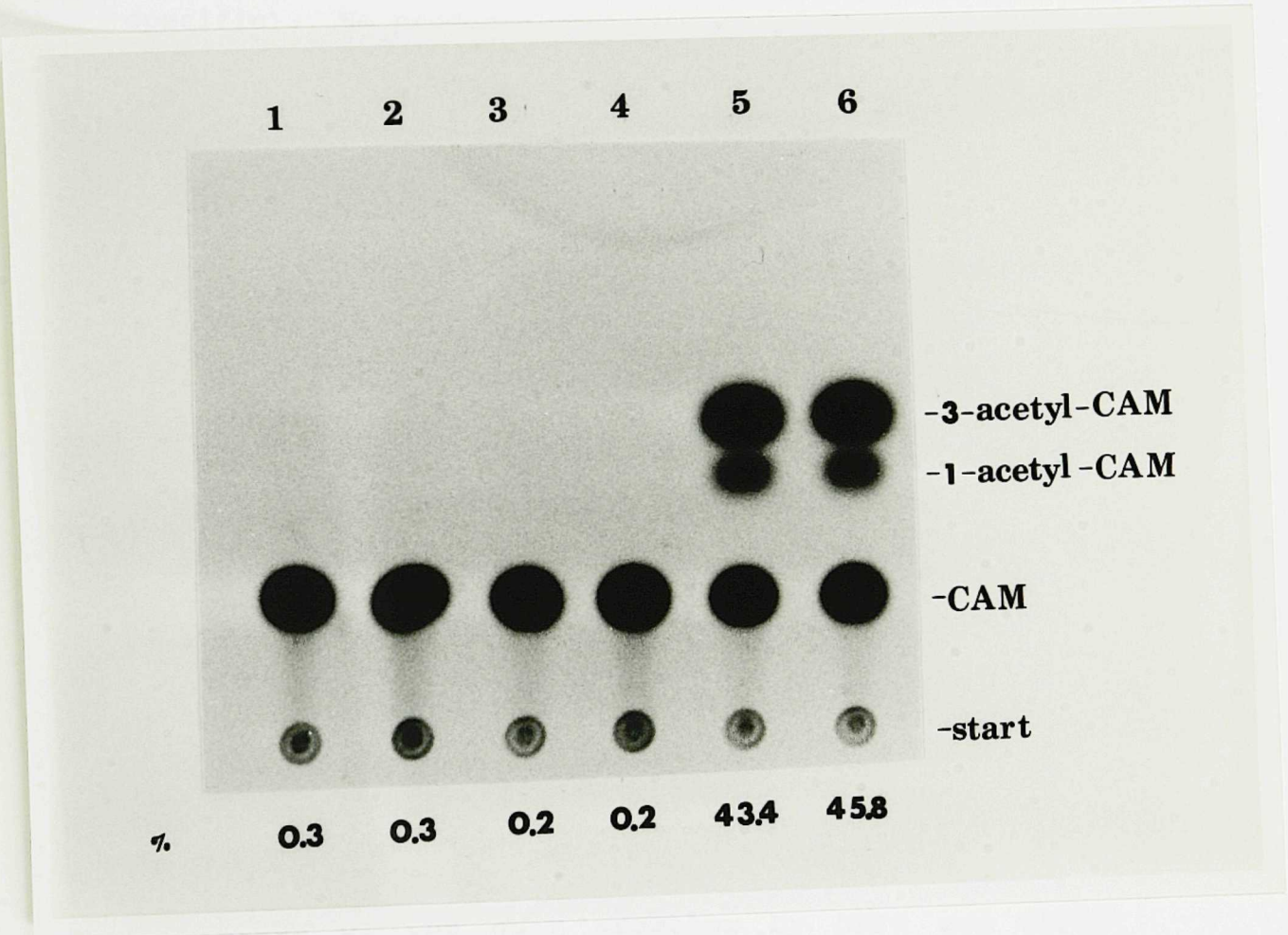

A
1 2 3 4 5
B
1 2 3 4 5
C
1 2 3 4 5
-1,3 diacetyl-CAM
-3 acetyl-CAM
-1 acetyl-CAM
-CAM
-start
0.5 0.5 0.3 0.6 94.6
0.5 2.5 2.3 92.0 97.0
0.5 2.3 3.1 84.0 97.0 %

Determination of promoter activity from gene 67 upstream sequences: Having mapped the 5' terminus of the gene 67 1.65kb major transcript (probably 5' co-terminal with the 3.6kb transcript) we were able to locate likely upstream regulatory sequences that might act as a promoter. The appendix contains a schematic of the upstream sequences that were cloned in front of a CAT gene (G4-3) in the vector pCAT3M, the conventional vector for testing putative promoter sequences in transient assays. As a control, a construct was made containing gene 67 promoter sequences in the opposite orientation (G4-2). These constructs were transfected into HFF cells and either left untreated or infected 1 day post-infection and harvested 3 days post-transfection. Cell extracts were made and tested for CAT activity. Figure 26 shows a typical assay. Both negative controls or the G4-3 construct had little detectable activity in untreated cells (figure 26 lanes 1 and 4). Transfected cells that were infected with VZV showed a marked increase (greater than 200-fold) in CAT activity with the G4-3 construct (figure 26, lanes 5 and 6) but not with the negative control (G4-2) (figure 26, lanes 2 and 3). We conclude from this that the gpIV promoter like the gpV promoter, requires VZV or VZV induced factors for optimal activity.

In order to confirm that the increase in CAT activity was due to an associated increase in RNA levels, and also that transcription initiation from the reporter gene promoter fusion was similar to that found in the virus, we carried out

FIGURE 26

Transactivation of the gpIV promoter by VZV infection. A) G4-3 (lanes 4-6) and G4-2 (lanes 1-3) plasmids were transfected into HFF cells and 3 days later harvested and cell extracts were made. Cells extracts were then assayed for CAT activity, spotted on silica gel plates, and chromatographed. In lanes 2 and 5 cells were infected with VZV Oka, and lanes 3 and 6 VZV Scott 24 hrs. post-transfection with a ratio of VZV-infected to uninfected cells of 1 to 10. Lanes 1 and 4 were left untreated. The percentage acetylation is listed below the respective lanes.

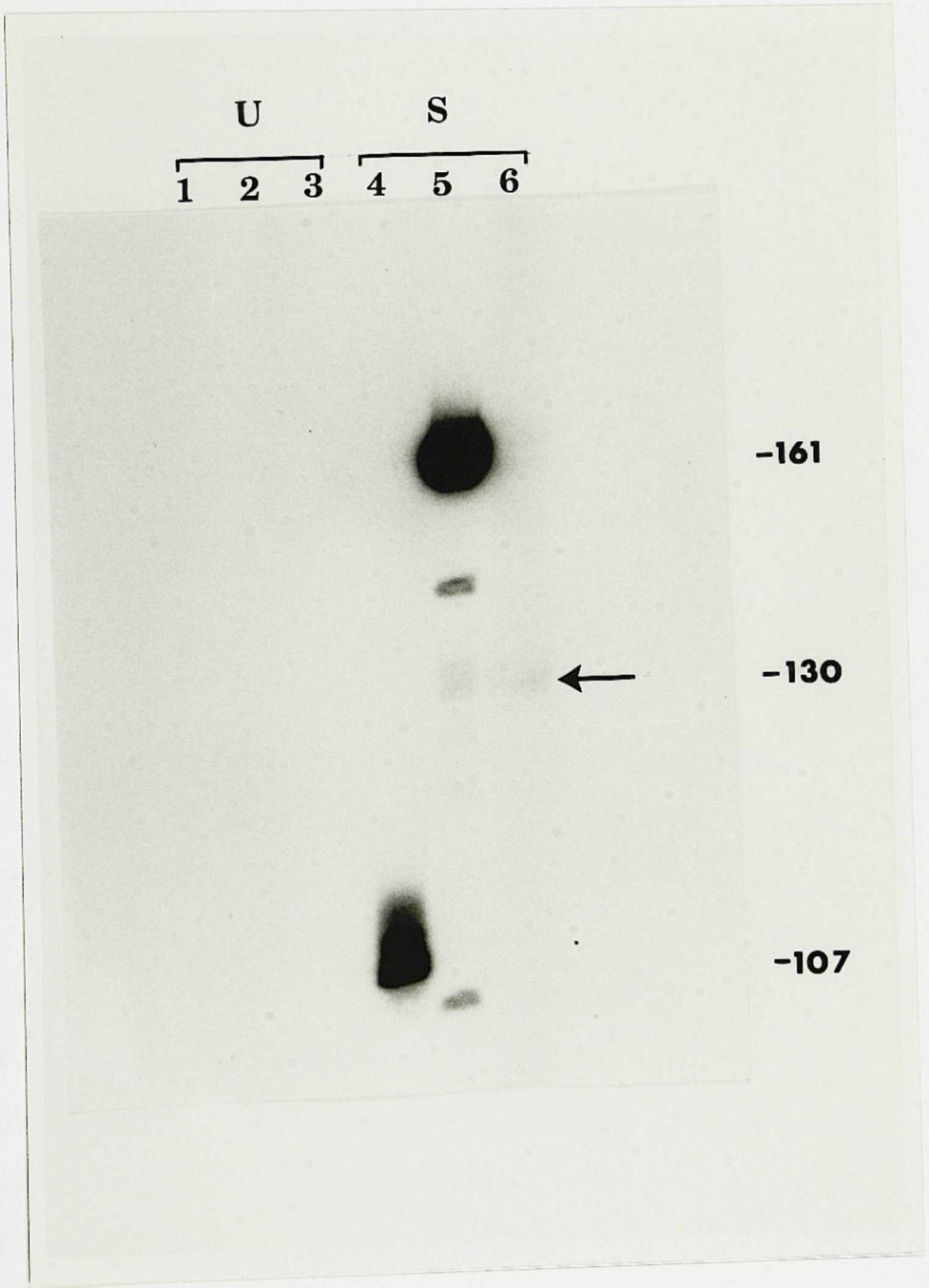

1
2
3
4
5
6
-3-acetyl-CAM
-1-acetyl-CAM
-CAM
-start
%
0.3
0.3
0.2
0.2
43.4
45.8

primer extension analyses on RNA from the transient assays. Figure 27 shows primer extension products from cell cultures transfected with G4-3 (lanes 1-3) and G4-3 with VZV infection (lanes 4-6). Three different oligonucleotides were used, OPL1 (gpV-specific), OPL5 (gpIV-specific), OPL15 (CAT gene-specific). No products were observed in uninfected cells. In transfected/infected cells, both OPL1 and OPL5 give 107bp and 161bp products as expected (discussed previously) from the infecting virus. In addition, a 130bp product was detected from OPL15 (figure 27, lane 6). This indicates that the G4-3 transcript is initiating faithfully since the construct contains 63bp of leader from VZV, 46bp from pCAT3M and the oligonucleotide is itself 20bp in size giving a total of 129bp. In addition, this indicates that CAT activity correlates with increased levels of RNA. A similar analysis was attempted for the gpV promoter constructs, however, no RNA was detected due to low levels of expression from this promoter. (This is probably due to technical problems since the gpIV CAT RNA was barely detectable, and transient assays showed this promoter to be >100-fold more active.)

Activation of the gpIV promoter by ORF4 and ORF62: Previous work by other laboratories have shown that VZV ORF62 and ORF4 can activate the VZV thymidine kinase and the gpI promoters (Inchauspe et al., 1989a). These proteins also bear some homology to HSV-1 Vmw175 and Vmw63 proteins that have been well characterized regulatory proteins (Davison and Scott, 1986b; McGeoch et al.,1986 and 1987). Having defined

FIGURE 27

Primer extension analysis on RNA from transient assays. RNA was harvested from HFF cells transfected with the G4-3 plasmid with (lanes 3-6) and without (lanes 1-3) VZV infection 72 hrs. post-transfection. ^{32}P-labeled primers OPL1 (lanes 1 and 4), OPL5 (lanes 2 and 5), and OPL15 (lanes 3 and 6) were hybridized with 20 µg RNA and a cDNA product was synthesized with reverse transcriptase. The products were resolved on an 8% denaturing polyacrylamide sequencing gel dried and autoradiographed. Numbers on the right are sizes of primer extension products in bases.


U
S
1 2 3 4 5 6
-161
-130
-107

that this promoter was VZV specific, and that VZV was necessary for activation, we attempted to dissect some of the probable VZV activators that might be responsible for this activity. Figure 28 shows G4-3 cotransfected with either ORF4 or ORF62 alone or the two genes together. Little or no activity is seen with ORF4 alone (figure 28, lane 1), while ORF62 has significant stimulation in comparison (figure 28, lane 2). Maximum activity was observed when both plasmids are present (figure 28, lane 3). These results are in general agreement with the work of other laboratories (Inchauspe et al., 1989b; Shapira et al., 1988). Similar analyses were carried out for the gpV promoter constructs, however no activation was observed. The reasons for this are described in the discussion.

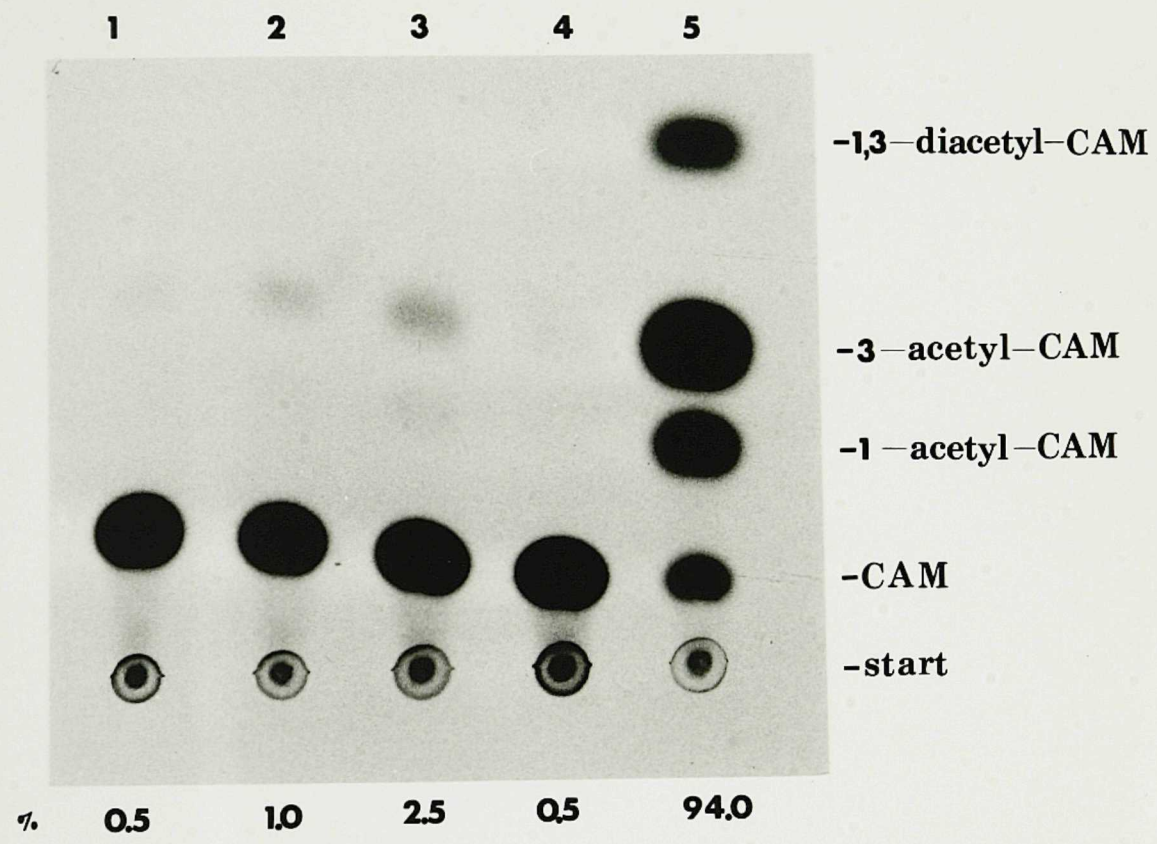
Characterization of protein products encoded by gpV.

Now that the transcripts mapping to ORF14 for both VZV strains Scott and Oka had been analyzed extensively, it was important to determine if the gpV protein products made from these strains 1) existed in VZV-infected cells and 2) reflected the levels of transcripts found in Scott and Oka.

To identify the protein products of ORF14, we used anti-VZV antibodies present in human sera to detect polypeptides expressed from vaccinia virus recombinants. We have also used antisera to recombinants as specific reagents to examine the properties of gpV in VZV-infected cells.

FIGURE 28

Activation of the gene 67 promoter with ORF4 and ORF62. HFF cells were transfected with G4-3 alone (lane 4) or cotransfected with G4-3 and the activating genes. Lane 1 contains G4-3 and ORF4, lane 2 G4-3 and ORF62, lane 3 G4-3 and ORF4 and ORF62 together. In all cases, 10 μg G4-3 was used and 5 μg each of the activating plasmids. Lane 5 is the CAT activity from 10 μg pSV_2 transfected cells from the same experiment. The numbers underneath the figure are percent conversion from CAM to acetylated forms.


1
2
3
4
5
-1,3-diacetyl-CAM
-3-acetyl-CAM
-1-acetyl-CAM
-CAM
-start
%
0.5
1.0
2.5
0.5
94.0

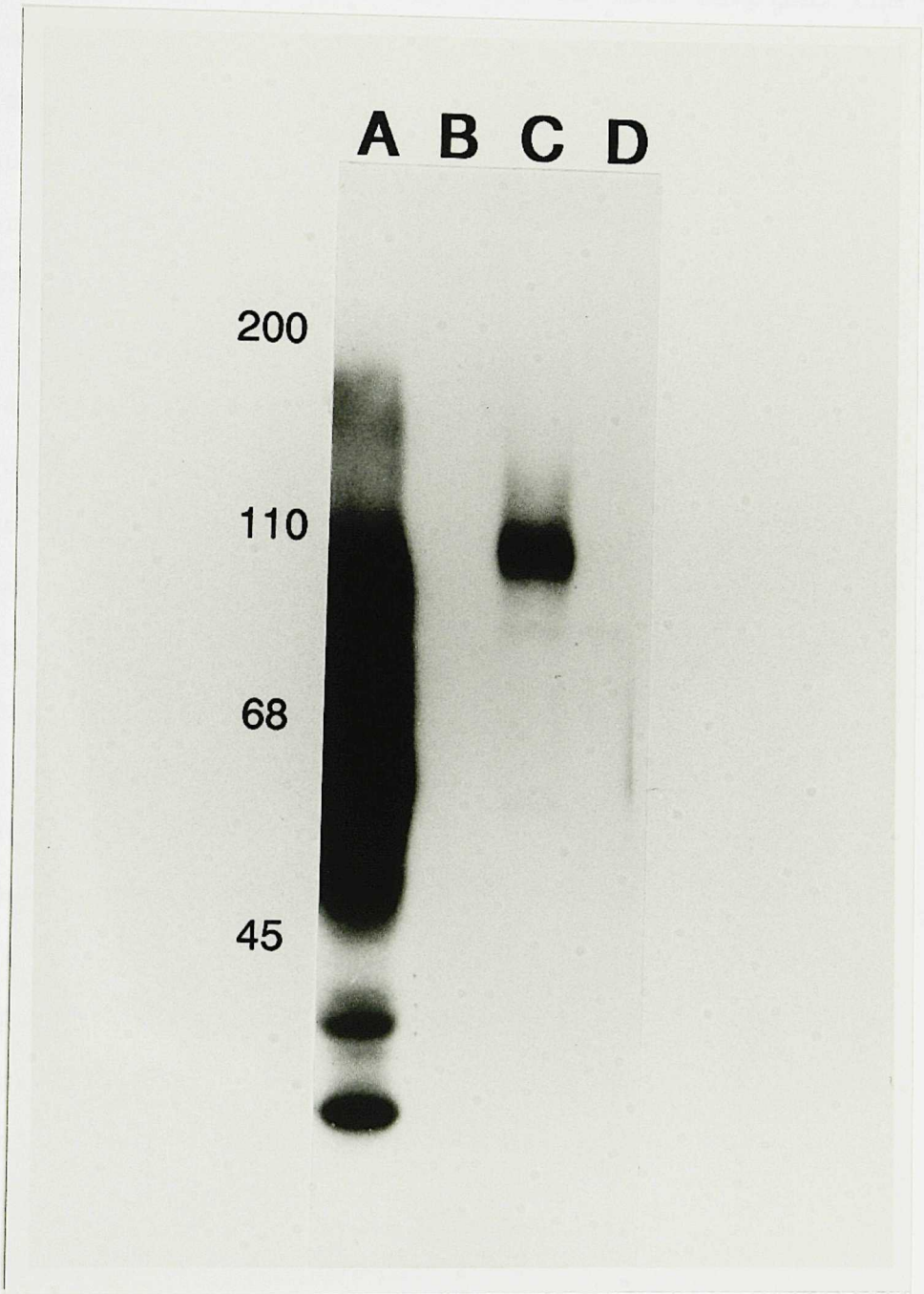
Construction of vaccinia recombinants expressing gpV: The plasmids and viruses used for generating vaccinia recombinants are shown in the appendix and described in the materials and methods. Each recombinant was checked to be tk^- and β-gal positive, then triple plaque-purified.

Expression from gpV-vaccinia virus recombinants: HFF cells were infected with the VZV gpV vaccinia recombinants and western blots of infected cells were prepared and reacted with human hyperimmune zoster antisera obtained from patients recovering from VZV infections. Figure 29 shows the reactivity of a zoster convalescent antiserum with blots derived from equivalent quantities of HFF cells infected with VZV Scott (lane A), uninfected cells (lane B), the vKIP5 vaccinia recombinant (lane C) and vSC8, a vaccinia recombinant containing vSC8 sequences, but no VZV DNA (lane D). The antiserum clearly reacts specifically with polypeptides present in the VZV-infected, but not uninfected, cell extracts. The vaccinia virus recombinant containing the gpV gene expressed proteins of 95-105K which reacted specifically with this and several other convalescent sera, indicating that the proteins expressed from VZV gene 14 are significant immunogens in natural VZV infection. These reactive polypeptides must be products of ORF 14, since the control virus, vSC8, expressed no polypeptides reactive with the VZV convalescent sera.

Rabbits were inoculated with vKIP5 to generate antiserum against gpV, and this serum was used to probe the cellular

FIGURE 29

Analysis of polypeptides from a vaccinia-gpV recombinant. Extracts of cells infected with VZV strain Scott (lane A), uninfected cells (lane B), and cells infected with vaccinia virus recombinants vKIP5 (lane C) and vSC8 (lane D) were electrophoresed, transferred to nitrocellulose and reacted with a 1/100 dilution of human convalescent zoster serum. Bound antibodies were detected using ^{125}I-labelled staphylococcus protein A. This Figure shows the autoradiogram and gives approximate molecular weights in kDa of a mixture of marker proteins, to the left of lane 4.


A B C D
200
110
68
45

location of gpV by indirect immunofluorescence. The results are in Figure 30, and show two samples of VZV Scott-infected cells probed with anti-vKIP5 antiserum; in both samples, the fluorescence appears to be associated with the plasma membrane and to be "patchy" in many cells. Both pre-immune sera (Figure 30c) and uninfected cells (Figure 30d) give faint overall background fluorescence.

Modification of this protein was also examined for addition of carbohydrate based on the prediction from sequence analysis that this was a glycoprotein. Several recent studies in our laboratory using glycosylation inhibitors such as tunicamycin, and enzymes that trim off carbohydrate residues, such as endoglycosidase H and F, have demonstrated that gpV is both N and O-linked glycosylated. The resulting carbohydrate modifications add approximately 30-40K to the apparent molecular weight of 95-105K gpV polypeptide (data not shown).

Comparison of gpV expression from VZV Scott and Oka-infected cells: Figure 31 shows a western blot derived from uninfected cells (M) or cells infected with VZV strains Oka (O) or Scott (S). The blot was probed with human VZV convalescent serum. Approximately 15-20 polypeptide species ranging in mol. wt. from 40 to 200K are found specifically in both VZV Scott (S) and VZV Oka infected cells (O). Interestingly, however, the patterns for Oka and Scott are not identical (see arrows in Figure 31a) and a consistent difference is seen around 95-105K; this is the size of gpV.

FIGURE 30



Indirect immunofluorescence in VZV-infected cells using gpV-specific antiserum. HFF cells infected with VZV strain Scott at two days post infection were treated with rabbit anti-vKIP5 antiserum followed by rhodamine-conjugated goat anti-rabbit serum. Shown are (A, B) two samples of VZV-infected cells treated with anti-vKIP serum, infected cells with pre-immune rabbit serum (C) and uninfected cells with vKIP5 serum (D).

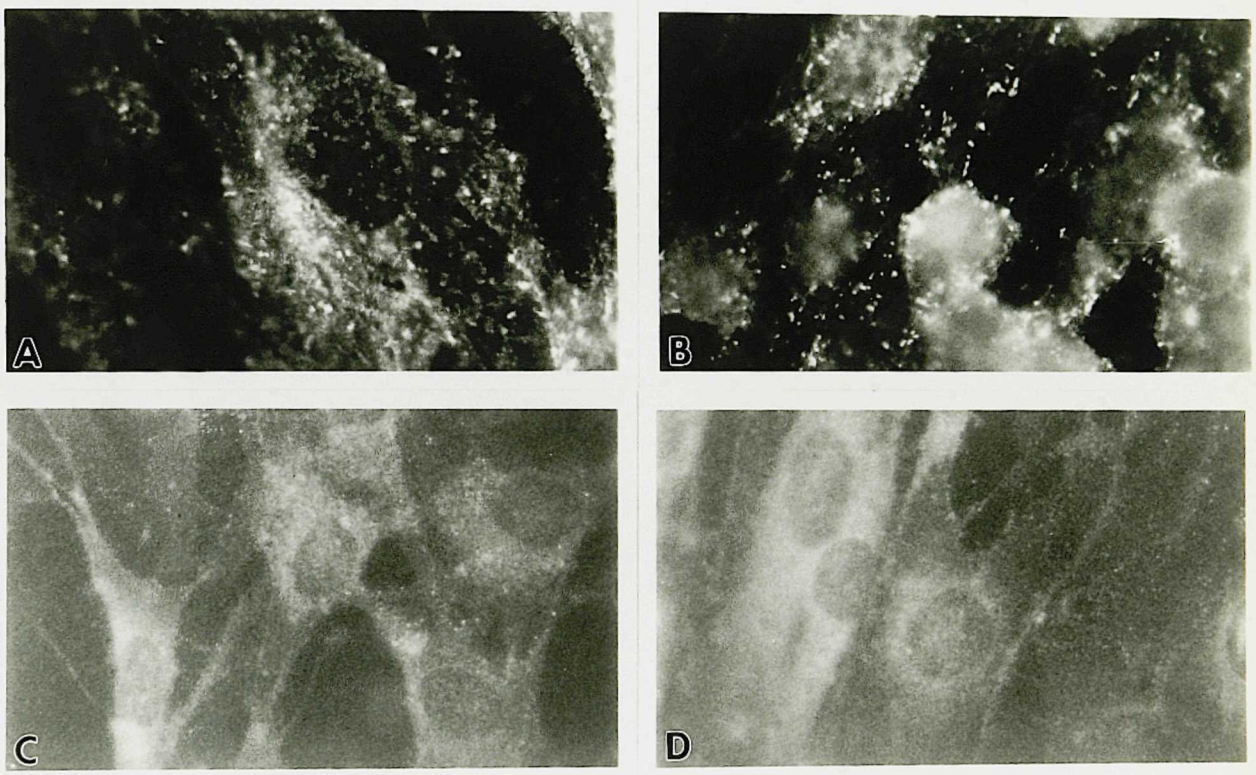

A
B
C
D

FIGURE 31

Western blots of infected cells using human convalescent antisera (a) blots were probed with human convalescent antisera that was untreated; (b) antisera absorbed with antigen from cells infected with VZV Scott; (c) antisera absorbed with antigen from cells infected with VZV Oka. Each blot contains extracts from uninfected cells (M) or cells infected with VZV Scott (S) or Oka (O). Approximate molecular wieghts for marker proteins are shown to the left of the figure in kDa.


a
b
c
M S O M S O M S O
200
110
68
45

If this is indeed gpV, either Oka makes a smaller gpV than Scott (as we would predict from the smaller size of the Oka ORF) which is masked on the blot and/or Oka makes only small amounts of gpV. To address these questions, we absorbed out antibodies from the human convalescent serum with a large excess of either Scott-infected or Oka-infected cell antigen and tested the residual antibodies in western blots. Figure 31b shows the reactivity of residual antibodies after absorption with Scott-infected cells; no reactive species are detected. In contrast, absorption with Oka-infected cells leaves antibodies reactive with one major species specific to Scott (Figure 31c, arrow), which is the same size as gpV; other more minor species were inconsistently seen. To verify that this absorbed-out antibody is directed at gpV, it was used to probe western blots of cells infected with the vaccinia ORF14 recombinant, vKIP5. The results (Figure 32) show that both the vKIP5 recombinant (lane b) and Scott-infected cells (lane d) give analogous reactivity at 95-105K, strongly suggesting that Oka-infected cell extracts contain very little gpV. As further confirmation of this deficiency in Oka, antiserum was prepared from rabbits infected with the vKIP5 and used as a specific probe for gpV. Identical western blots of Scott, Oka and uninfected cell polypeptides were reacted with either a VZV convalescent human serum (Figure 33a) or with the anti-vKIP5 serum (Figure 33b). As expected, anti-vKIP5 antibodies bound specifically to 95-105K polypeptides present in Scott-infected cells, but not in

FIGURE 32

Western blots of cell extracts. Polypeptides from cells infected with VZV Scott (d), VZV Oka (e), uninfected cells (c), wild-type vaccinia (a) or the vaccinia-gpV virus recombinant vKIP5 (b) were separated on SDS-PAGE. Western blots were reacted with 1:100 dilutions of human sera absorbed with excess antigen from cells infected with VZV Oka as described in the text. The approximate molecular weights of a mixture of marker polypeptides are shown in kDa on the right.

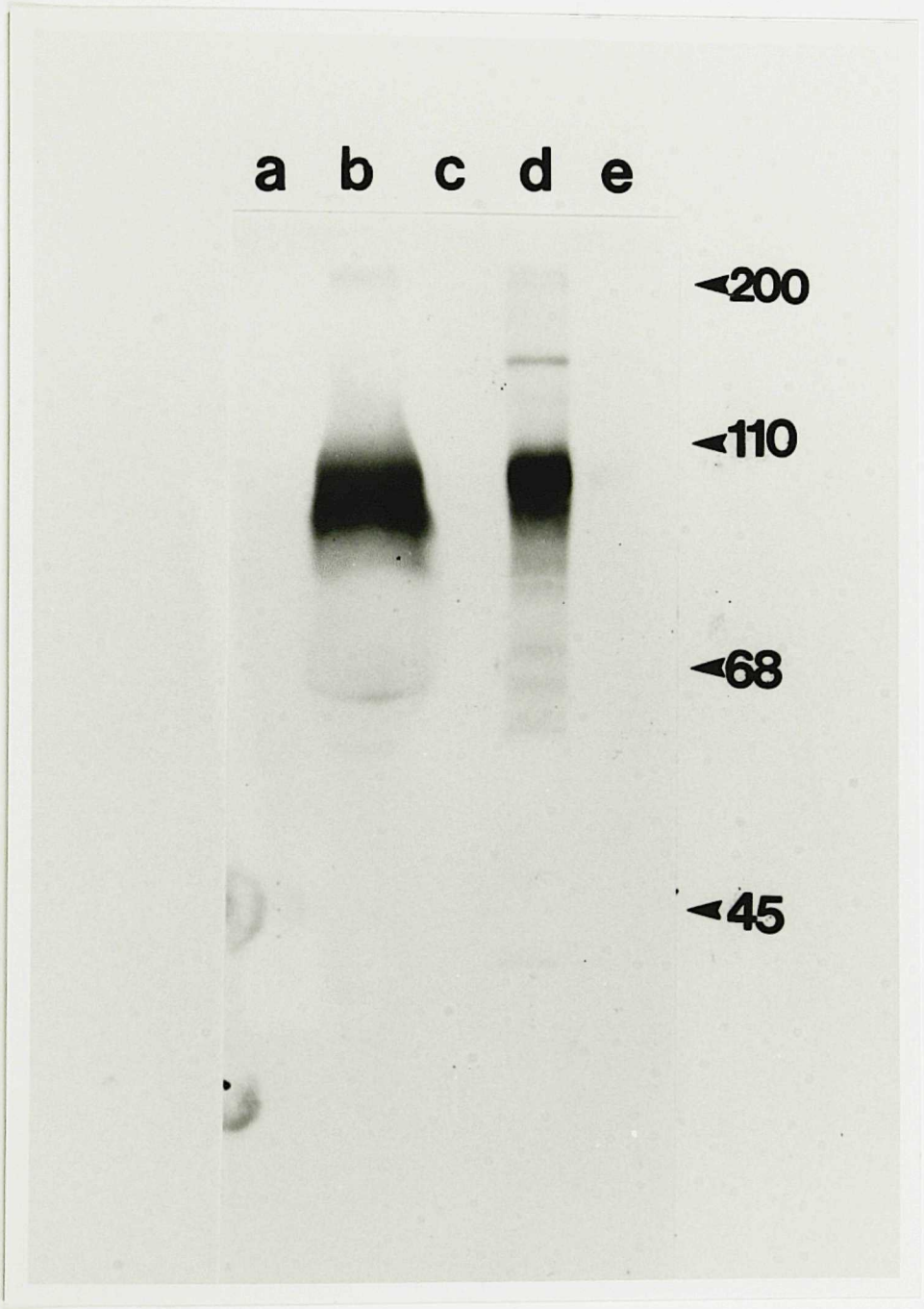
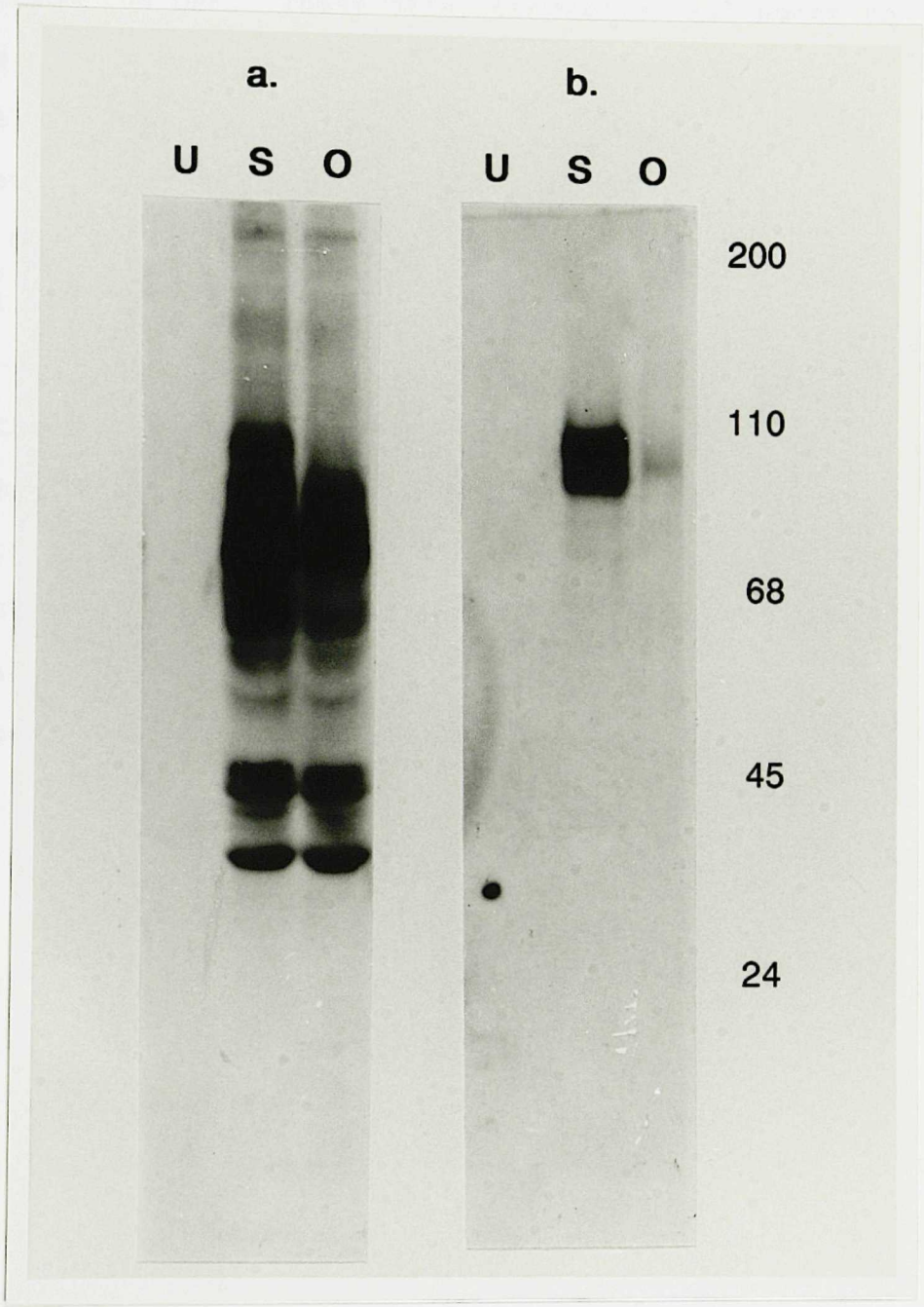

a b c d e
200
110
68
45

FIGURE 33

Western blots of VZV-infected cell proteins. Uninfected HFF cell (U), and Scott (S) and Oka (O) infected cell proteins were separated by PAGE and blotted to nitrocellulose. In panel (a), blots were reacted with a VZV convalescent human serum, while in panel (b) blots were reacted with rabbit anti-gpV antiserum. In both cases, reaction was detected with ^{125}I staphyloccocus protein A. The Figure shows the autoradiograms, with molecular weights of protein markers given in kDa to the right of panel (b).


a.
U S O
b.
U S O
200
110
68
45
24

uninfected cells. In VZV Oka-infected cells, however, only trace amounts of gpV are visible, of the smaller size expected from the ORF. These data, therefore, all point to defective accumulation of gpV in VZV Oka-infected cells. Thus, the amounts of protein correlate well with the levels of RNA detected. This is also compatible with our hypothesis that the Oka vaccine strain defect in gpV synthesis is at the RNA level.

Characterization of the proteins encoded by gpIV.

Unlike ORF14, there was previous evidence that glycoprotein products (45K and 55K) were made from ORF67 in VZV-infected cells Davison et al., 1985). These reacted specifically with an anti-oligopeptide serum generated against sequences derived from ORF67, encoding gpIV. In addition, Vafai et al. (1988) identified a monoclonal antibody as well as rabbit polyclonal sera generated against *in vitro* translation products made from ORF67, and those reacted with polypeptide products of 45K to 60K in VZV-infected cells. These investigators also showed that some precursor forms of gpIV were phosphorylated, but not mature processed products. We have used similar methodology to that described above for identification and characterization of the gpV polypeptide, to confirm the size and identity of the gpIV polypeptide. This gives us a unique and useful reagent to assess some of the functional properties of gpIV.

Construction of vaccinia recombinants expressing gpIV: The construction of a shuttle vector containing the gpIV ORF and recombination of this plasmid into vaccinia virus is described in the materials and methods and shown in the appendix. Each recombinant was checked to be tk^- and β-gal positive, then triple plaque-purified. We then tested the vaccinia recombinant for production of a gpIV protein product.

Expression from gpIV-vaccinia virus recombinants: HFF cells infected with the gpIV vaccinia recombinants were lysed and prepared for SDS-PAGE electrophoresis. Western blots were then performed to detect gpIV protein in these lysates. The same VZV-convelescent antiserum used for detection of gpV in previous studies was used in this experiment. As shown in figure 34, the detected proteins were found only in extracts from vaccinia recombinants carrying the gpIV ORF (clone g4c) in the correct orientation (lane D). Lanes containing vSC8 (no gpIV, lane E) and g4w (gpIV in the wrong orientation, lane C) did not react with this antisera. The proteins detected were two species, approximately 45K and a heterogenous 50-60K species. The reactive proteins must be from ORF67 since the g4w control (ORF67 in the opposite orientation, e.g., antisense to the p7.5 vaccinia promoter) and vSC8 do not react with this antiserum.

The gpIV recombinant was then injected into rabbits and antisera was recovered. This anti-g4c serum was unable to detect any proteins on a western blot containing VZV-infected

FIGURE 34

Analysis of polypeptides from a vaccinia-gpIV recombinant. Extracts of cells infected with VZV strain Scott (lane A), uninfected cells (lane B), and cells infected with vaccinia virus recombinants g4w (lane C), vSC8 (lane E) and g4c (lane D) were electrophoresed, transferred to nitrocellulose and reacted with a 1/100 dilution of human convalescent zoster serum. Bound antibodies were detected using ^{125}I-labelled staphylococcus protein A. This figure shows the autoradiogram and gives approximate molecular weights in kDa of major bands appearing in lane D.


A
B
C
D
E
-60
-45

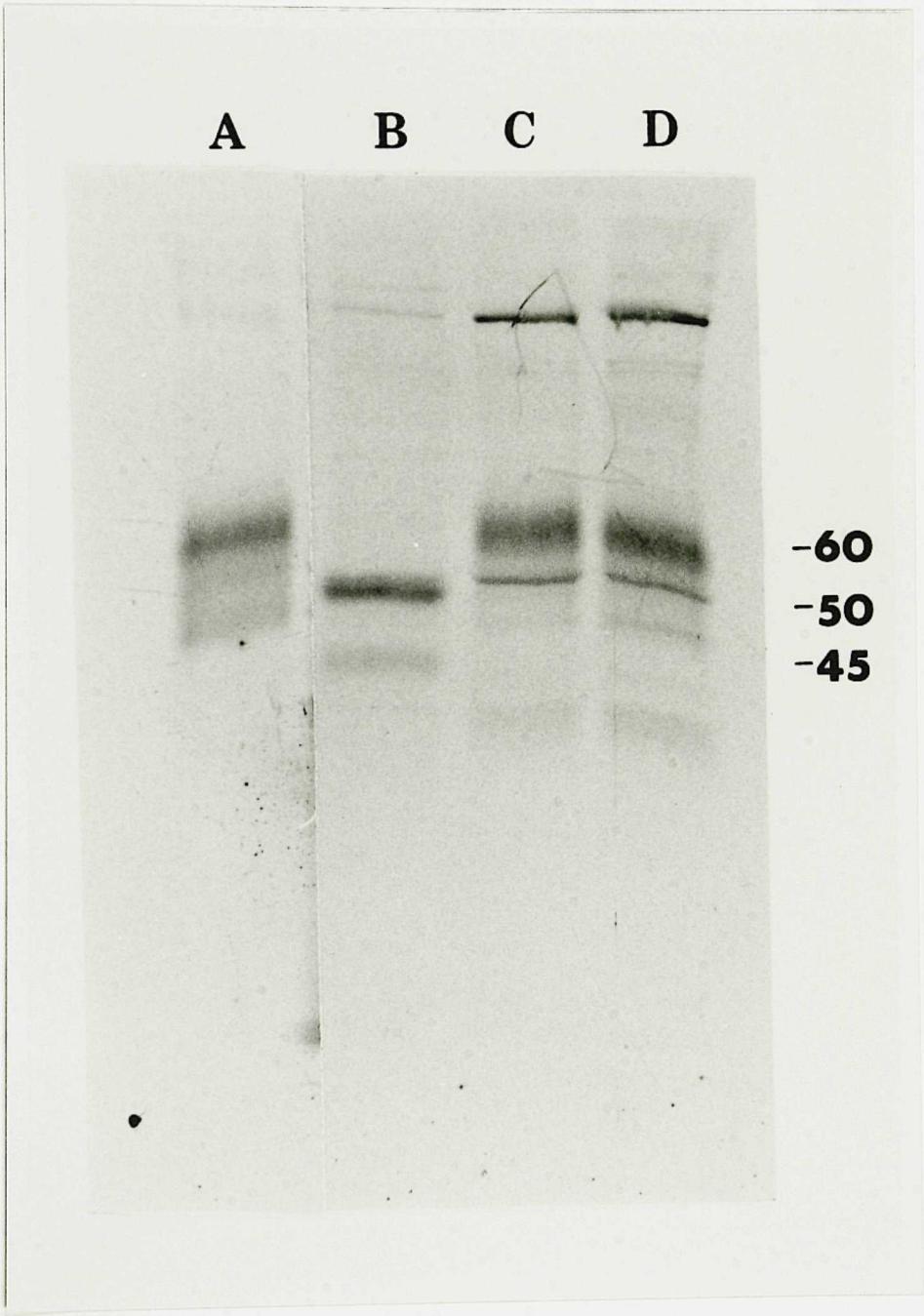
cell lysates. However, VZV-infected cell extracts labeled with ^{35}S-methionine were able to be immunoprecipitated from a pulse chase experiment with the anti-g4c antisera. Figure 35 shows major polypeptides of about 50K in a pulse and about 60K in a chase that reacted specifically with this antisera (lanes B-D). Smaller, less abundant species of 39K and 45K were also seen. Uninfected cell lysates showed no reactivity (data not shown). These results correlate very well with our western blot data and that of other laboratories (Davison et al., 1985; Vafai et al., 1988). In addition, it also suggests that the immune-reactive epitopes in the previous western blot experiments need to be in their natural conformation, and may explain the weak reactivity of the human antisera in western blots against the g4c recombinant. In order to confirm that gpIV was indeed a glycoprotein, we labeled VZV infected cells with ^{14}C-glucosamine. Extracts from these cells were immunoprecipitated with the rabbit anti-g4c antiserum. As seen in figure 35, specific products of approximately 45K and 60K were recognized by this antisera (lane A). No reactivity was observed in ^{14}C-glucosamine labeled uninfected cell extracts (data not shown).

Functional activity of the gpV and gpIV polypeptides

VZV neutralizing activity in gpV and gpIV-specific antsera: We have been able to demonstrate, using purified virion preparations, that gpV is present in the VZV particle (J. Hougland and P.R. Kinchington, unpublished observations).

FIGURE 35

Immunoprecipitation of radioactively labeled VZV infected cell extracts using gpIV specific antiserum. Cells were labeled with ^{14}C-glucosamine (lane A), or ^{35}S-methionine, lanes (B-D, where C and D are duplicate samples) and precipitated with rabbit anti-g4c antisera. Lane B is an extract from labeled VZV-infected cells pulsed for 15 min. and lanes B and D are extracts from cells similar to lane B only chased with cold methionine for 4 hr. Samples were electrophoresed on SDS-PAGE gels, dried and autoradiographed. Sizes of major bands are listed in kDa to the right of the figure.


A
B
C
D
-60
-50
-45

This raises the possibility, which could be tested with the recombinant-derived rabbit antiserum, that gpV might serve as a VZV-neutralizing antigen. We also decided to test whether gpIV antiserum also contained neutralizing activity. The antisera was mixed with cell free VZV preparations and plated on HFF cells for infectivity assays. After four days, cells were fixed and plaques counted; for comparison, both rabbit pre-immune sera and a human zoster convalescent serum were used in the assay. As can be seen in Table 2, preimmune serum had a slight effect on VZV infectivity, at the lowest dilution. In contrast, both human serum and gpV-specific rabbit serum substantially affected the infectivity of VZV Scott, reducing the titres by greater than 90% at dilutions up to 1/25 and by more than 50% at a dilution of 1/125. The gpIV-specific anti-sera also reduced the titres by 80% at dilutions up to 1/25 but only reduced titres by 10% at dilutions of 1/125. Inclusion of guinea pig complement with anti-gpV or anti-gpIV antiserum in the assay had no effect on neutralization of VZV. As predicted, VZV Oka was neutralized substantially less well than Scott by the anti-gpV serum, whereas human serum and the anti-gpIV serum neutralizes both viruses to similar extents. These results imply that gpIV and gpV are targets for complement-independent virus-neutralizing antibodies and that the level of neutralization of VZV Oka reflects small amounts of gpV in VZV Oka virions.

Detection of C3bi binding activity for gpV: The gpV homologue in HSV-1, gC, has been demonstrated to have C3bi

Table 2

Antiserum	Serum dilution 1:5	1:25	1:125
Preimmune serum	70*	85	100 (Scott)
	70	86	100 (Oka)
Human serum	0**	8	36 (Scott)
	0	0	16 (Oka)
Anti-gpV serum	0	13	36 (Scott)
	19	76	100 (Oka)
Anti-gpIV serum	13	22	90 (Scott)
	4	19	111 (Oka)

*Numbers are the percentage survival of plaques relative to a control plate untrteated with serum.

**0 means that no plaques were visible.

Dilutions of antisera were mixed with preparations of VZV-infected cells containing about 100pfu of virus. After 2hr. at room temperature, the mixtures were plated on HFF cells and fixed after six days.

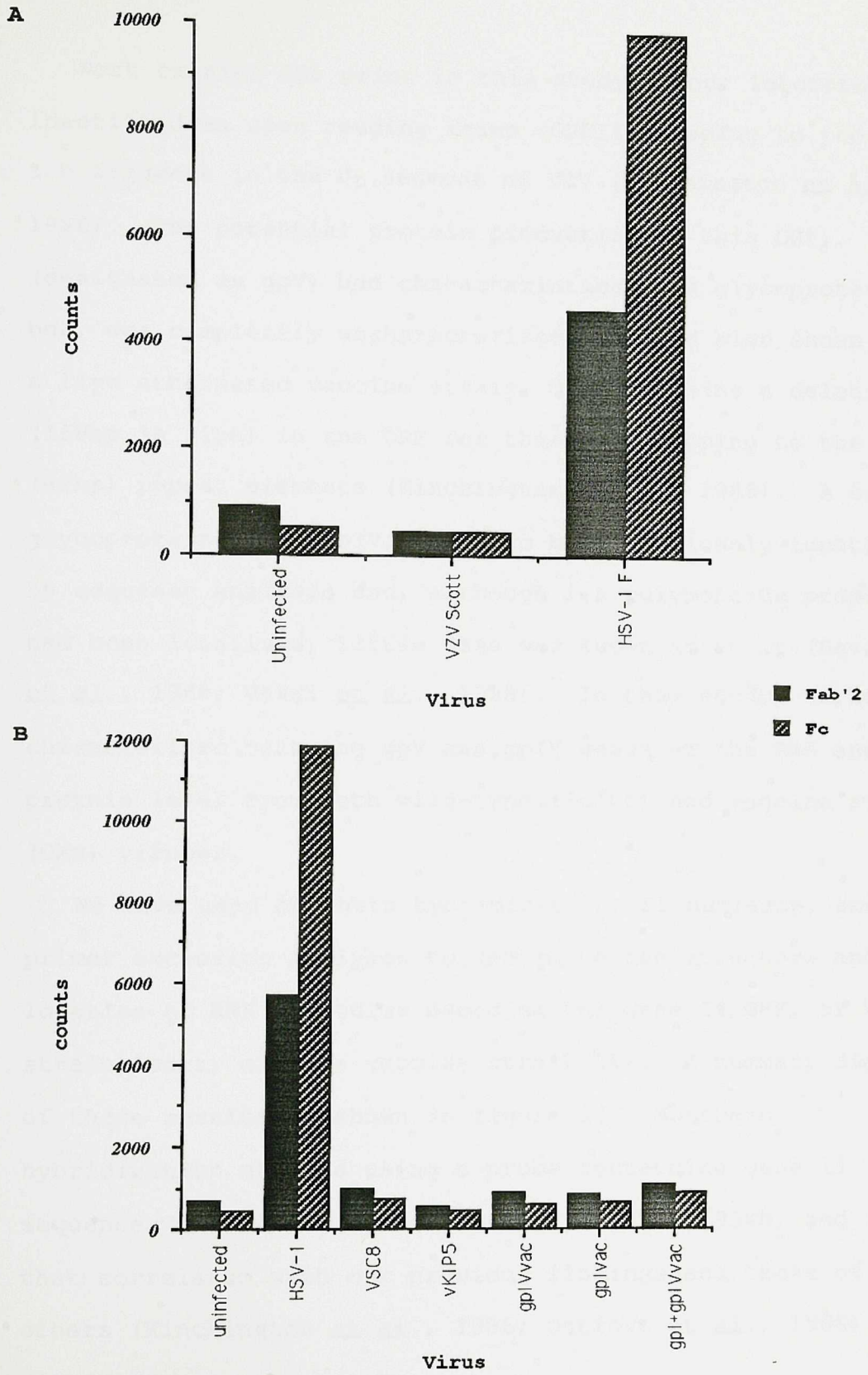
binding capabilities. In collaboration with Dr. Gary Cohen at the university of Pennsylvania Medical School, we tested the ability of VZV and the vKIP5 vaccinia recombinant to bind the C3bi complement component. Sonicated preparations from cell extracts of HSV-1, VZV or vKIP5 were bound to nitrocellulose. ^{125}I-labeled C3bi was then incubated with these nitrocellulose strips, but only HSV-1 lanes were reactive with the radiolabeled C3bi. No reactivity was observed in VZV or vKip5 infected cell extracts (unpublished observations). We concluded from this study (repeated several times) that VZV (and gpV in particular) does not have a C3bi binding capacity under standard assay conditions.

Detection of Fc binding activity associated with gpIV: The gpIV homologue in HSV-1 is glycoprotein I (gI) (Davison et al., 1986b; McGeoch et al., 1986). gI has been demonstrated to be involved in a complex with HSV-1 glycoprotein (gE) to form an Fc receptor, using vaccinia recombinants expressing the gE and gI glycoproteins (Bell et al., 1990). There are conflicting reports on whether VZV possesses Fc binding activity (Asano and Tkahashi, 1980; Edson et al., 1980; Gelbet et al., 1981; Okata and Shigeta, 1979). To test this we constructed a vaccinia recombinant that expresses the gpI gene product that is homologous to the gE protein product in HSV-1 for use in coinfection with the gpIV vaccinia recombinant. Using the assay for Fc binding (described in materials and methods) that other laboratories have employed for detection of HSV-1 Fc binding activity, we

examined the ability of these vaccinia recombinants to bind ^{125}I-labeled IgG Fc and Fab'2 fragments (Johnson et al., 1988). As can be clearly seen in figure 36, only the HSV-1 infected cells had Fc binding capability (figure 36 A and B). VZV infected cells, the g4c recombinant, the gpI recombinant, or cells coinfected with the gpI and gpIV recombinants did not display Fc binding over backround (uninfected cell) levels (figure 36 A and B). We suggest from this data that VZV does not possess an Fc binding capacity.

FIGURE 36

Fc receptor activity in HSV-1 and VZV glycoprotein recombinant vaccinia viruses. Monolayers of HFF cells were infected with HSV-1 F, VZV Scott, the gpIV vaccinia recombinant, the gpI vaccinia recombinant or coinfected with the gpI and gpIV recombinants. After 14 hrs. the cells were washed and the Fc assay was carried out according to the materials and methods. The results shown are an average of three determinations. ^{125}I-labeled Fab'2 fragments were also included as a control. Hatched bars represent binding to Fc fragments, and solid bars represent binding of Fab'2 fragments.


A
10000
8000
6000
4000
2000
0
Counts
Uninfected
VZV Scott
HSV-1 F
Virus
Fab'2
Fc
B
12000
10000
8000
6000
4000
2000
0
counts
uninfected
HSV-1
VSC8
vKIP5
gplVvac
gplvac
gpl+gplVvac
Virus

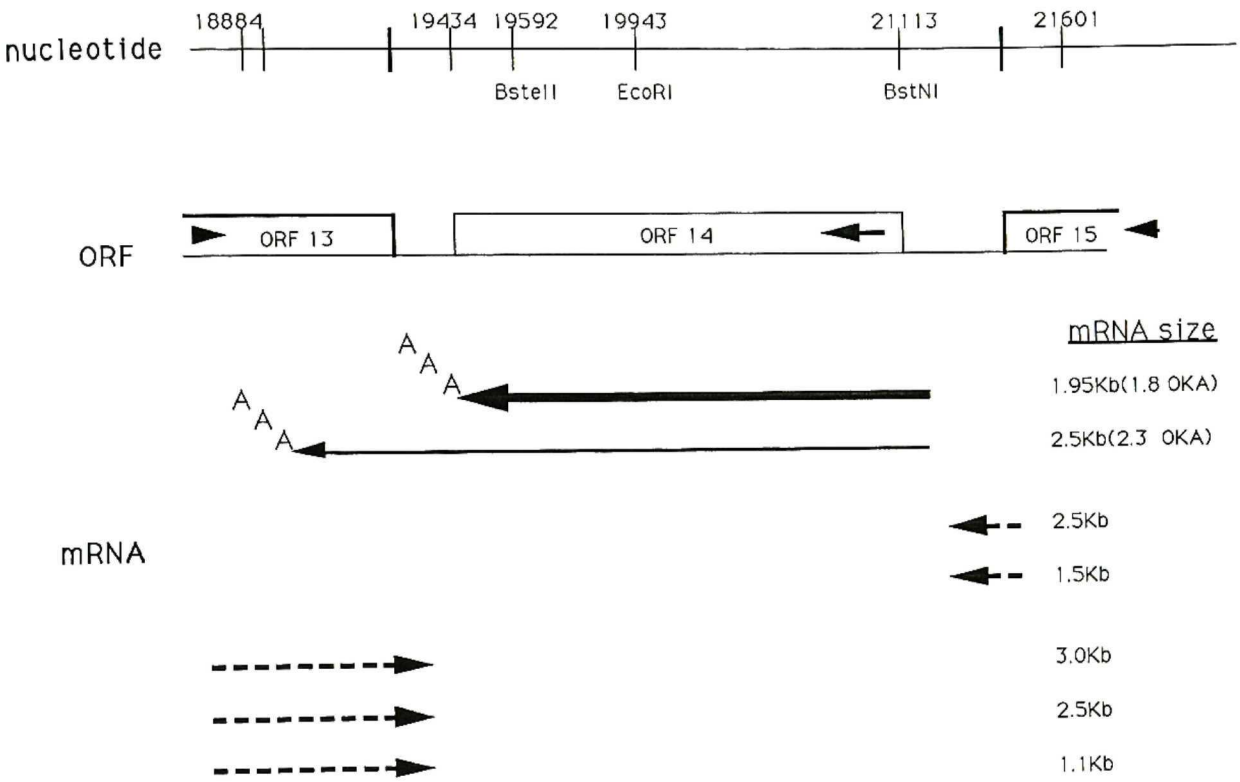
Discussion

Work carried out prior to this study in our laboratory had identified an open reading frame (ORF14) mapping to the EcoR I P fragment in the U_L segment of VZV (Kinchington et al., 1986). The potential protein product(s) of this ORF, (designated as gpV) had characteristics of a glycoprotein but, was completely uncharacterized. We had also shown that a live attenuated vaccine strain, Oka, contains a deletion (168bp in size) in the ORF for this gene mapping to the R2 (42bp) repeat elements (Kinchington et al., 1986). A second glycoprotein gene, gpIV, had also been previously identified by sequence analysis and, although its polypeptide products had been localized, little else was known about it (Davison et al., 1985; Vafai et al., 1988). In this study, we have characterized both the gpV and gpIV genes at the RNA and the protein level from both wild-type (Scott) and vaccine strain (Oka) viruses.

We have used northern hybridization, S1 nuclease, and primer extension analyses to determine the structure and location of RNA molecules encoding the gene 14 ORF, of VZV strain Scott, and the vaccine strain Oka. A summary diagram of these results is shown in figure 37. Northern hybridization studies using a probe containing gene 14 sequences detected 3 transcripts of 2.5kb, 1.95kb, and 1.5kb that correlated with our previous findings and those of others (Kinchington et al., 1986; Ostrove et al., 1985).

FIGURE 37

The gene 14 transcription map. The scale at the top indicates the gene 14 region of VZV DNA with important restriction sites. Below is the location of ORFs, with arrows showing the direction of transcription. The lower part of the figure shows the major and minor transcripts for gene 14 and the locations of other RNA species mapping upstream and downstream of gene 14. The sizes in kilobases of each transcript are indicated in the column on the right.


nucleotide
18884
19434
19592
19943
21113
21601
BsteII
EcoRI
BstNI
ORF
ORF 13
ORF 14
ORF 15
mRNA size
1.95Kb(1.8 OKA)
2.5Kb(2.3 OKA)
mRNA
2.5Kb
1.5Kb
3.0Kb
2.5Kb
1.1Kb

Several other probes in northern hybridization studies containing both upstream and downstream sequences to gene 14 indicated that 2 of these transcripts are specific for gpV and are synthesized in VZV-infected cells. For strain Scott, these are 2.5kb and 1.95kb transcripts, whereas Oka contains 2.3kb and 1.8kb transcripts. These, and all viral specific transcripts detected from the BamH I F region of the VZV genome were polyadenylated. The determination that Oka synthesizes smaller transcripts agrees with our previous studies showing that Oka contains 3.5 fewer copies of the 42bp repeats, adding up to a total of 168bp (Kinchington et al., 1986). Interestingly, the level of steady state transcription for gpV transcripts in strain Oka is up to 26-fold less than found in Scott-infected cells as quantitated by densitometry from northern blot autoradiograms.

S1 nuclease analysis as well as primer extension analysis localized the 5' ends of these transcripts to -36bp to -37bp from the initiation codon found at 21113bp (see Figures 16 and 37). As mentioned previously, we favor initiation at -37bp since most mRNAs synthesized by cellular polymerase II initiate at a purine (reviewed in McKnight and Tjian, 1986). Under the conditions used, no splicing was detected, and the results suggest that transcripts encoding gene 14 sequences were different at their 3' termini. Examination of sequences upstream from the cap site revealed a potential AT-rich region (ATTTAAATT) that may serve as a "TATA" box for the promoter at -25 to -33bp. Further upstream at -55bp is a

consensus CAT box (CCAATT). The positioning of these elements is consistent with other eukaryotic promoters, but their role in VZV gene expression remains to be established (reviewed in Mitchell and Tjian, 1989; Maniatis et al., 1987; McKnight and Tjian, 1986). The length of the 5' untranslated region for gene 14 is quite short (37bp), in comparison with the only other VZV genes that have been mapped, the thymidine kinase gene (420bp) and ORF62 (71bp), the HSV Vmw175 homologue (Davison and Scott, 1986b; McKee et al., 1990). Like the thymidine kinase and ORF62 genes, several consensus "TATA" regions reside upstream of gene 14, a TTATAATT at -145bp, and a TTATAATT at -371bp. Thus, the simple examination of upstream sequences cannot be used for prediction of likely promoter regions and transcription initiation in VZV. It is also of interest to note that the first possible initiation codon in ORF14, at -80bp, is not present in gene 14 transcripts and that, therefore, the second ATG is exclusively used; in any case, if the first ATG were used, the N-terminal sequence of gpV would not resemble a signal sequence. We have also shown that the shorter ORF (second ATG) protein products (21113bp to 19434bp) expressed in a vaccinia virus expression system appear to be identical to gpV products made in infected cells (see results and below).

The 3' termini of gene 14 transcripts have also been defined; that of the 1.95kb (1.8kb in Oka) transcript is approximately 10bp downstream from the termination codon, and

an ATAAA sequence. The end of the 2.5kb transcript (2.3kb in Oka) is about 440bp downstream from the termination codon, close to ATAATT and ACGTAAA sequences. While all of these sequences deviate somewhat from ATTAAA and AATAAA polyadenylation consensus motifs, they bear some similarity to other deviant signals identified in herpesviruses. The HSV-1 gC 3' co-terminal family, for example, terminates 10-20bp downstream of ATAAAA, while the VZV thymidine kinase 3' terminus is just downstream of AGTAAA (Frink et al., 1981; Davison et al., 1986b). The deviation from normal eukaryotic consensus polyadenylation signals may be important for the regulation of transcription termination and polyadenylation in VZV. Recent studies in HSV-1 have demonstrated a role for additional GU-rich sequences downstream of the polyadenylation signal that may be important for efficient and proper termination (Mclaughlin et al., 1985). We have noted such sequences in VZV (see results section), but their role remains to be verified.

The data in this report describe a transcription pattern that is quite different from that found for HSV-1 gC, the apparent homologue to VZV gene 14 (Frink et al., 1981). Glycoprotein C is encoded in an unspliced 2520bp mRNA that is part of a spliced family of transcripts sharing a portion of the leader of the major unspliced mRNA. These transcripts are part of a 3'co-terminal family of transcripts that also include a small transcript controlled by its own promoter. Given the apparent similarity between gC and gpV, it is

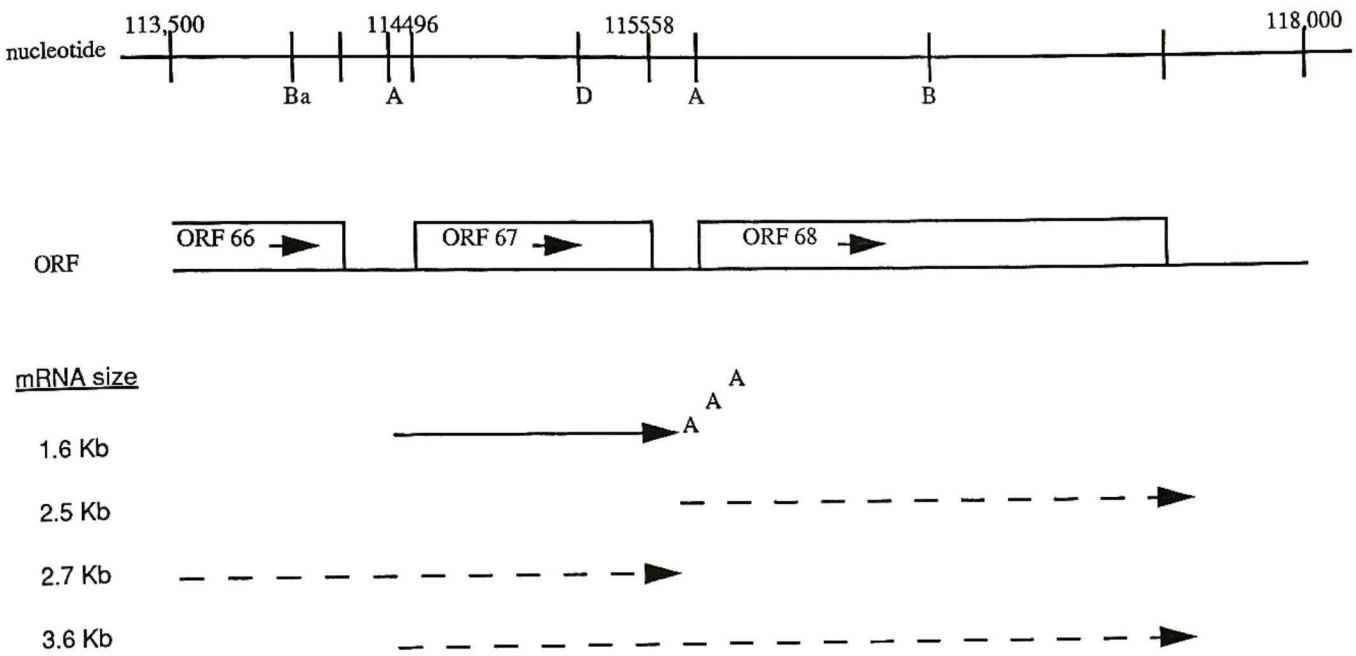
perhaps surprising that their transcript patterns have evolved differently. The close similarity of gene arrangement in HSV and VZV (particularly in U_L) would make it seem likely that the pattern of gene expression in the two viruses would also be similar. While the data currently available are not extensive, the posibility that there exist substantial differences in transcription between these two alphaherpesviruses now needs to be considered.

In this context, Homa et al.(1988) have shown that a 15bp fragment 5'-GGGTATAAATTCCGG-3' from the gC promoter was able to confer specific late gene regulation. This fragment also contains the putative 'TATA' region. Comparison of this sequence to the -25bp region from the VZV gpV cap site reveals a sequence 5'-TCATTTAAATTCCGC-3' that is very similar to that found for HSV-1 gC. These investigators have also demonstrated that this fragment is a "movable" entity, that can confer promoter activity similar to the wild-type promoter in a heterologous background. It would be of interest to test the analogous sequence in the gpV promoter for these same properties.

We have used mapping techniques similar to those described for gene 14 analysis to map transcripts for gene 67, encoding glycoprotein IV (gpIV). The results are summarized in Figure 38. Earlier work described transcripts mapping to the general region of the VZV genome around ORF67 (Ostrove et al., 1986; Kato et al., 1989; Reinhold et al., 1988). In this report, we defined the termini of the major 1.65kb

FIGURE 38

The transcripts mapping to gene 67. This diagram shows the nucleotide number on the top line with horizontal lines labeled where pertinent restriction enzyme sites are located. Ba; BamHI, A; AccI, D; DdeI, B; BstEII. The middle part of the diagram shows locations of open reading frames in this region. The bottom part of the diagram shows the location of transcripts found mapping to this region of the genome. Sizes of transcripts are listed on the left side of the diagram in kilobases.


nucleotide
113,500
114496
115558
118,000
Ba
A
D
A
B
ORF
ORF 66
ORF 67
ORF 68
mRNA size
1.6 Kb
2.5 Kb
2.7 Kb
3.6 Kb
A
A
A

transcript encoding ORF67 as detected by northern hybridization and standard RNA mapping techniques. Northern analysis by us and by other laboratories gave evidence that the major 1.65kb transcript encoded gpIV and that the 2.7 and 3.6kb transcripts were readthrough transcripts. Northern and primer extension analyses revealed that transcripts mapping to gene 67 sequences were identical for the Scott and Oka strains. Subsequent S1 and primer extension analyses gave a precise location of the 5' terminus of the 1.65kb transcript 91bp upstream of the initiation codon, giving a leader sequence significantly longer than the gpV leader. Likely eukaryotic promoter elements have been identified, an AT-rich region at -25bp that may serve as a TATA box, and a CAT box motif at -65bp. The 3' terminus of this transcript was localized 23bp downstream of two overlapping poly(A) sequence signals, ATTAAATAAA.

This information, when compared to other transcript data from another VZV structural gene (gpV) showed similarities and differences. Both genes exibited heterogenous start sites, at which both transcripts might initiate at a pyrimidine nucleotide and not the more conventional purine nucleotide (see figures 16 and 22). Both genes also have atypical TATA box regions at -25bp, whereas many other canonical TATA sequences are located in various places upstream of the translational initiation codon. These data are consistent with the other published VZV transcript mapping data. Finally, both putative gene promoters have

ordinary CAT box motifs at approximately -55 to -65bp upstream of the capsite. Since only four genes have been mapped in VZV to date, more transcript mapping will have to be done to determine if particular characterisitics and patterns unique to VZV transcription will emerge. What is a common picture at this point is that prediction of potential regulatory sequences for VZV transcription initiation and termination may give false results and, therefore, they must be experimentally determined. An example of this is demonstrated by the VZV ORF62 gene (McKee et al., 1990). Preliminary characterization of VZV ORF62 indicated that the TATA box and further upstream regulatory sequences around 374bp from the first AUG, based on inspection of the nucleotide sequence (Felser et al., 1988). Recent RNA mapping results, however, place the ORF62 transcript 5' end at 71bp to the first AUG, making the likely TATA box (TTTAAA) 25-30bp upstream to this. Deletion analysis of the promoter element also substantiates this data by localizing promoter activity to a minimum 131bp upstream of the first AUG. In contrast to the 5' end analysis, the 3' termini of ORF67 maintains a more conventional result. The RNA mapping data shows a distinct 3' termini 23bp downstream of two overlapping poly(A) signals at 115617bp (see figures 23 and 38). The potential poly(A) signals observed for the gpV gene as well as the VZV dPyK gene are atypical, as mentioned before and have precedents in other systems (Donehower et al., 1981 Davison et al., 1986b; Frink et al., 1981. The

question of why VZV appears to use atypical control sequences for some genes (dPyK, gpV), and conventional ones in others (gpIV) is not clear (the 3' end for the ORF62 gene transcript has not been published).

Sequencing results showed that the putative upstream promoter sequences were equivalent for both Scott and Oka strains. In an attempt to address the mechanism of the Oka gene 14 transcriptional "defect", we tested the hypothesis that the 42bp repeating elements might act as enhancers. Under a variety of conditions, using a conventional enhancer test vector, we could not detect classical enhancer activity from these elements. These results indicate that a probable mechanism for the transcriptional defect in Oka resides in some transactivating protein(s). An alternative mechanism is that the 42bp repeating elements must be present in the proper context, ie, residing 3' to a compatible promoter element such as the native gpV promoter. A construct of this type could easily be made, using site directed mutagenesis to create a unique restriction site at the 3' end of the R2 repeat region. The whole gpV promoter with the R2 repeats could then be cut and religated as a fusion protein with a reporter gene such as β-galactosidase or CAT which could also be tested in a transient transfection assay system. Recently, however, our laboratory has obtained several VZV isolates from Japan that disply a wide range of size heterogeneity in the number of R2 repeats. These isolates all produce wild-type levels of gpV, suggesting that the

repeats may not function in regulating expression of gpV (unpublished observations).

In order to address some of the questions involved with potential transcription initiation processes involving viral encoded or viral induced factors, we cloned putative promoter sequences in front of the CAT gene, and tested them in transient assays. In the first set of experiments, we looked at the ability of upstream sequences from the gpV ORF to act as a promoter. The basal level of activity from this promoter was quite low, whereas VZV infection induced this promoter (and not control constructs) >5 fold (figure 25). In addition, the gpV promoter was 40 to 50-fold less active than the gpIV promoter (figure 25). This is curious since, *in vivo*, we detect similar levels of gpV and gpIV transcripts. There may be several reasons for this observation. Although the CAT reporter gene is widely used, in many cases it has been shown to generate a highly unstable transcript, which may be poorly translated in the context of certain regulatory sequences in eukaryotic cells. Using another reporter gene such as luciferase or β-galactosidase might alleviate these problems, and allow better determination of promoter activity. Also, as mentioned previously, the 42bp repeat elements may be required for optimal promoter activity. Alternatively, there may be a problem with the fact that we cloned a large fragment (+36 to -504bp) to test as the gpV promoter. It is possible that this fragment may be too large (or too small) and that there

are certain required sequences missing or deleterious sequences present that may affect expression from this promoter. Nested-set deletions from both the 5' and 3' ends of the BstN I/EcoR I promoter fragment (see Appendix for gpV promoter fusion constructs, S6 and O14) cloned back into the CAT reporter plasmid might help answer this question. Finally, we should also consider the possibility that this promoter may be inherently weak, and that gpV transcripts are extremely stable and accumulate throughout the course of infection. This would fit with our *in vivo* results, since we have analyzed RNA from cultures at late times of infection. Another important observation from these experiments is that both Scott and Oka infection induced the gpV promoter equivalently. If Oka contained a defective transactivator, or transactivating ability, we postulated that the transient assay would detect it. Possible reasons for not detecting the Oka defect in this assay are many, and may hinge on the observations and problems associated with the low levels of activity from the gpV promoter discussed previously. In addition, transient assays may not put regulatory elements in the proper context. For example, investigators studying the "late" expression observed for HSV gC found that separation of promoter elements from the viral genome in transient assays resulted in the promoter being regulated as an early gene (not sensitive to PAA) (Shapira et al., 1987). Only when promoter activity was measured directly on viral transcripts made from the gC promoter residing in the viral

genome was it regulated as a late gene (sensitive to PAA) (Homa et al., 1986a). As observed with gC, the manifestation of defective gpV transcription in Oka may be linked to its association within an actively replicating viral genome. In theory, this possibility could be resolved using classic marker rescue experiments, however, at this time, no such system that is technically feasible is available for VZV. One final possibility is worth considering. It is possible that Oka contains a *ts* mutation affecting gpV transcription. As mentioned previously, Oka has been demonstrated to have a slight temperature sensitive phenotype in an infectivity assay compared to the parent Oka strain as well as other wild-type strains (Takahashi, 1984). Technically, several manipulations are performed in transient assays allowing several temperature variations to occur. In the gpV promoter analysis, the transactivation by Oka may be affected by these temperature variations. It has been documented that for some HSV *ts* mutants, a short change to the permissive temperature during the growth cycle can result in loss of the ts phenotype in a particular experiment (Addison et al., 1984). The potential *ts* character of gpV synthesis in Oka could be readily tested on the virus itself by looking at gpV viral transcripts and proteins made under stringent temperature conditions *in vivo*.

The results from experiments described in Figures 27 and 28 indicate that the gpIV promoter has very low basal levels of activity, but that viral factors or viral induced factors

significantly activate expression of the CAT gene driven by these promoters. Primer extension analysis on RNA from transient assays also indicated that CAT activity was associated with increased levels of correctly-initiated RNA. Whether the increased levels of RNA are due to increased initiation of RNA synthesis or post-transcriptional stabilization is not known. In addition, effects on translational efficiency may also be involved. Nevertheless, it seems most likely that activation is a reflection of increased RNA synthesis. These results are in agreement with recent work by Inchauspe et al. (1989b), demonstrating that VZV infection can activate the VZV tk promoter to significant levels.

Having established the general boundaries of the gpIV promoter and its inducibility, specific questions can now be addressed, such as the specific sequence requirements for promoter activity. To address this, various deletion or linker-scanning mutations in the Acc I/BamH I fragment presently fused to CAT (see appendix) could be made and tested for loss or gain of promoter activity. These analyses might provide information for determination of important signals that could then be placed in a non-VZV backround promoter region to ascertain if VZV specificity is transferred. In addition, important sequences identified by these analyses might also be substituted into the gpV promoter constructs to determine if these are "movable" elements.

Once we determined that VZV infection activated the gpIV promoter, we attempted to dissect possible transactivating proteins encoded by the virus. Two genes, ORF4 and ORF62, have been previously shown to be activating proteins (Inchauspe et al., 1989a; Cabriac et al., 1990). In agreement with results published with other target promoters, we found that ORF4 had little activity on its own, but that ORF62 had some activating capacity. Maximum activity was observed when ORF4 and ORF62 were used together. The activation we observed, however, did not approach that reached using whole virus. One explanation for this is that activator gene polypeptides are synthesized in low amounts (they are controlled by their natural promoters), or that some autoregulatory events on the activator gene promoters are occurring that alter expression. Placing these activator genes in front of constitutive heterologous promoters would help to test this possibility. Another possible explanation is that there are other VZV encoded gene products required for optimal activation of this promoter. ORF61, which is the homologue to a general transactivator in HSV, Vmw110, may be one required gene product (Davison et al., 1986b; McGeoch et al., 1986). However, two independent reports have suggested that this gene product has poor transactivating properties and in one case actually had a negative regulatory effect (Inchauspe et al., 1989a; Cabriac et al., 1990). We are attempting to construct large cosmid clones containing the entire VZV genome, and to use these in co-transfection

experiments with the target promoter/CAT plasmids to delineate potential transactivating proteins in an approach similar to the procedure used by Challberg et al. (1986) to identify viral proteins required for DNA replication. In addition, Cabirac et al. (1990) have recently shown that the HSV-1 Vmw110 gene product in concert with the VZV ORF62 gene could transactivate the VZV tk promoter, to much higher levels than the ORF62 gene product alone (or with ORF61 and ORF62). Additional studies using various combinations of HSV and VZV transactivators may help to elucidate regulatory proteins that are both specific to and/or permiscuous with herpes virus (and other) promoters.

We also analyzed the polypeptide products encoded by the gene 14 and gene 67 ORFs. These studies were important for several reasons: 1) We identified for the first time gpV polypeptide products in infected cells and showed that these products were glycoproteins 2) We correlated Scott and Oka gpV protein accumulation with RNA levels 3) We confirmed the size and glycoprotein nature of the gpIV polypeptides 4) We tested for putative biological activity associated with the gpIV and gpV glycoproteins.

In this study we have identified products from ORF14 (gpV), as a set of polypeptides of about 95-105K, which are present in VZV particles. The sizes of proteins made from the gpV gene in vaccinia recombinant-infected cells are similar (95-105K) but, with vaccinia, the bias is towards the 95k range, while authentic VZV gpV is mainly around 105K.

This may reflect less or somewhat different glycosylation in vaccinia-infected cells. As discussed in the results section, we have now completed several experiments giving evidence that gpV contains N-linked glycosylation. Two approaches were used to investigate this: *in vivo* tunicamycin treatment and *in vitro* endoglycosidase degradation. These indirect methods had to be used because none of our anti-gpV antibodies works well in immunoprecipitation experiments. In the first approach, HFF cells infected with vKIP5 (see Appendix) were incubated with tunicamycin from 8 to 24 hrs. post infection and then harvested for western blot analysis with human zoster convalescent serum. The results show that tunicamycin, which inhibits N-linked glycosylation, significantly reduces the formation of 95-105 kDa polypeptides, revealing two novel polypeptides of 64 and 58 kDa and two minor polypeptides of 60 and 68 kDa. We interpret this result to suggest that the 58 kDa polypeptide represents unmodified gene 14 product (the predicted molecular weight based on predicted amino acid composition), while the other species are partially modified. In the second series of experiments, gpV was isolated from vKIP5-infected cells by elution from polyacrylamide gels, and incubated *in vitro* with endoglycosidase F (endo F), which trims both N-linked high mannose simple and low mannose complex oligosaccharides. Incubation products were concentrated by TCA precipitation, blotted to nitrocellulose after SDS-PAGE, and probed using a convelescent human serum.

The results showed that gpV is trimmed from 95-105 kDa to 85-90 KDa by endo F under both high pH (endoglycosidase activity plus peptido-N-glycosidase activity) and low pH (endoglycosidase activity) buffered conditions. Together with the tunicamycin results, these data show that the products of VZV gene 14 are glycosylated and contain at least N-linked mannose-type unsialated oligosaccharides. In addition, the digestion with endo F of N-linked sugars from mature species results in novel polypeptides significantly larger than the predicted size of unglycosylated protein; thus, some glycosylation may be resistant to endoglycosidase F, and one possibility is this is O-linked. Many herpesvirus glycoproteins are O-glycosylated, including HSV-1 gC, to which gpV is partially related. As mentioned previously, other VZV glycoproteins such as gpI are modified with fatty acids, or phosphorylated (Edson et al., 1987; Harper and Kangro, 1990). The mature gpV polypeptide may also be modified by these processes.

We also find that the gpV polypeptide product made by the vaccine strain Oka is smaller in size and abundance than in other VZV strains. The Oka 168bp deletion in ORF14 correlates to about 6K of predicted amino acid sequence, and this shows up in the differences in apparent molecular weights. The abundance of Oka gpV polypeptide product was estimated to be about 2% of the wild-type Scott. Thus, it appears that the vaccine strain Oka accumulates very small amounts of gpV polypeptide product, and this can be related

to a decreased amount of transcript observed in infected cells. In order to confirm that the small amounts of gpV associated with Oka was not due to a lack of epitopes reactive with our antiserum, or that Oka gpV is an unstable protein, we recently have expressed the Oka gpV protein in vaccinia virus (recent unpublished observations). Rabbit antisera generated against this recombinant reacted in western blots just like anti-vKIP5 antisera. In addition, the protein as expressed by vaccinia was made in equal amounts to that for vKIP5, indicating that the Oka gpV ORF is fully functional and that the polypeptide is inherently a stable protein.

Mutants in HSV-1 containing defects in synthesis of gC (the homologue to gpV) are dispensible for growth of this virus in tissue culture (Homa et al., 1986b). It is tempting to speculate that gpV might also be nonessential for VZV growth in tissue culture. Creation of null mutants that are totally gpV$^-$ (Oka makes a little gpV) would answer this question, although as mentioned before, generation of VZV recombinants is technically difficult (only one published account has been reported). However we do consider one strategy technically feasible. It would involve generation of a plasmid containing gpV sequences flanking a β-galactosidase gene under a VZV promoter (gpIV for example). The plasmid would then be transfected with infectious VZV DNA. Homologous recombination between the gpV sequences in the plasmid and gpV sequences in viral DNA would result in

insertional inactivation of the viral gpV gene and aquisition of a β-galactosidase gene under a VZV promoter. An overlay containing X-gal on infected cell monolayers would detect blue plaques that could then be picked and purified.

Another feature which gpV shares with HSV gC is its intracellular location. Indirect immunofluorescence with anti-vKIP5 antisera shows "patchy" plasma membrane fluorescence in VZV-infected cells, reminiscent of that shown by anti-HSV gC sera in HSV-infected cells (Eberle and Courtney, 1980). This fluorescence pattern may indicate the association of gpV with itself, or with other cellular or viral encoded products.

A similar methodology used to identify ORF14 gene products was used to characterize ORF67 gene products. We have identified gene products from gene 67 vaccinia recombinants and VZV-infected cells that range in size from 45-60K. Sugar labeling studies have also indicated that this protein is modified with carbohydrates. These results are in agreement with results obtained by other laboratories, preliminarily characterizing this protein (Davison et al., 1985; Vafai et al., 1988). The nature and number of other post-translational modifications is still unclear at this time.

Many herpesvirus glycoproteins are targets for neutralization by circulating antibodies. We show here that anti-gpV and anti-gpIV antibodies are able to neutralize VZV in the absence of complement, suggesting that they are exposed on the virion envelope; in addition, we demonstrate

that gpV and gpIV are natural immunogens, since antibodies to these proteins are present in sera from VZV convalescent patients. In this sense, gpV is similar to its other herpesvirus homologs; e.g. the HSV gC and the pseudorabies virus gpIII are major neutralization targets. While these studies suggest that gpIV and gpV are on virion particles, more detailed studies involving immune electron microscopy on virion particles, and western blot or immune precipitation on purified virions will have to be performed to define the presence in virions. Other investigators have identified a glycoprotein of 45K to 60K in virion preparations, however the specificity of their antibodies at that time was unknown (Okuno et al., 1983). In addition Namazue et al. (1986) also showed that a monoclonal antibody that recognized a series of proteins from 92-45Kd was able to neutralize VZV in the prescence of complement. It is likely that their antibody may have recognized the gpIV polypeptide. We consider our studies more definitive since the nature of our antigen (g4c vaccinia recombinant) and specificity of our antibodies is more definitive than in previous studies.

In contrast to the similarity to HSV-1 of the ability of VZV glycoprotein homologues to induce neutralizing antibodies, these proteins do not have the same immune evasion functions; gpV does not possess C3bi binding activity, and gpIV (or gpI) does not seem to have Fc binding capacity. This is not entirely surprising since the amino acid homology shared between these glycoproteins is small

compared to other homologous glycoproteins like VZV gpII and HSV gB. In addition, it is unknown if these immune evasion mechanisms play a role in the pathogenesis of HSV. Interestingly, the gpIV equivalent in HSV, gI, is also nonessential for growth in tissue culture (Longnecker et al., 1987). This may also be the case for VZV, and could be tested in a manner similar to that described for gpV.

References

Addison, C., F.J. Rixon, J.W. Palfreyman, M. O'Hara, and V.G. Preston. 1984. Characterization of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virol. 138: 246-259.

Almeida, J.D., A.F. Howatson, M.G. Williams. 1962. Morphology of varicella (chickenpox) virus. Virol. 16: 353-355.

Arvin, A.M., C.M. Koropchak, and A.E. Wittek. 1983. Immunologic evidence of reinfection with varicella-zoster virus. J. Infect. Dis. 148: 200-205.

Asano, Y., and M. Takahashi. 1979. Studies on the polypeptides of varicella-zoster (V-Z) virus. I. Detection of varicella-zoster virus polypeptides in infected cells. Biken J. 22: 81-89.

Asano, Y., and M. Takahashi. 1980. Studies on the polypeptides of varicella-zoster (V-Z) virus. II. Synthesis of viral polypeptides in infected cells. Biken J. 23: 95-106.

Ausubel, F.M., R. Brent,and R.E. Kingston. 1987. Current protocols in molecular biology. New York: Wiley-Interscience.

Bachenheimer, S.L., and B. Roizman. 1972. Ribonucleic acid synthesis in cells infected with herpes simplex virus. VI. Polyadenylic acid sequences in viral messenger riboucleic acid. J. Virol. 10: 875-879.

Bartkoski, M.J. Jr., and B. Roizman. 1976. RNA synthesis in cells infected with herpes simplex virus. XIII. Differences in the methylation patterns of viral RNA during the reproductive cycle. J. Virol. 20: 583-588.

Berger, S.Y., and A.R. Kimmel. 1987. Guide to molecular cloning techniques. Acedemic Press Inc., San Deigo, California.

Berk, A.J. and P.A. Sharp. 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. USA 75: 1274-1278.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-264.

Brunell, P.A., A.A. Gershon, W.T. Hughes, H.D. Riley, and J. Smith. 1972. Prevention of varicella in high risk children: a collaborative study. Pediatr. 50: 718-722.

Brunell, P.A., A.A. Gershon, S.A. Uduman, and S. Steinberg. 1975. Varicella-zoster immunoglobulins during varicella, latency, and zoster. J. Infect. Dis. 132: 49-54.

Brunell, P.A. 1983. Fetal and neonatal varicella-zoster infections. Semin Perinatol. 7:47-56.

Bullowa, J.G.M. and S.M. Wishik. 1935. Complications of varicella. I. Their occurance among 2,534 patients. Am. J. Dis. Child 49:923-926.

Burke, B.L., R.W. Steele, O.W. Beard, J.S. Wood, T.D. Cain, and D.J. Marmer. 1982. Immune responses to varicella-zoster in the aged. Arch. Intern. Med. 142: 291-293.

Cabirac, G.F., R. Mahalingam, M. Wellish, and D.H. Gilden. 1990. Trans-activation of viral tk promoters by proteins encoded by varicella zoster virus open reading frames 61 and 62. Vir. Res. 15: 57-68.

Challberg, M.D.. 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Nat. Acad. Sci. USA 83: 9094-9098.

Chirgwin, J. M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5299.

Costanzo, F. G. Campadelli-Fiume, L. Foa-Tomas, and E. Cassai. 1977. Evidence that herpes simplex DNA is transcribed by cellular poymerase II. J. Virol. 21: 996-1001.

Davison, A.J.,and J.E. Scott. 1983. Molecular cloning of the varicella-zoster virus genome and derivation of six restriction endonuclease maps. J. Gen. Virol. 64: 1811-1814.

Davison, A.J., D.J. Waters, and C.M. Edson. 1985. Identification of the products of a varicella zoster virus glycoprotein gene. J. Gen. Virol. 66: 2237-2242.

Davison, A.J., C.M. Edson, R.W. Ellis, B. Forghani, D. Gilden, C. Grose, P.M. Keller, A. Vafai, Z. Wroblewska and K. Yamamishi. 1986a. New common nomenclature for the glycoprotein genes of varicella zoster virus and their glycosylated products. J. Virol. 57: 1195-1197.

Davison, A.J. and J.E. Scott. 1986b. The complete DNA sequence of varicella zoster virus. J. Gen. Virol. 67: 1759-1816.

Deluca, N.A. and P.A. Schaffer. 1988. Physical and functional domains of the herpes simplex virus transcritpional regulatory protein ICP4. J. Virol. 62: 732-743.

Donehower, L.A., L. Huang, and G.L. Hager. 1981. Regulatory and coding potential of the mouse mamary tumor virus long terminal redundancy. J. Virol. 37: 226-238.

Dumas, A.M., J.L.M.C. Geelen, W. Maris, and J. vander Noordaa. 1980. Infectivity and molecular weight of varicella zoster virus DNA. J. Gen. Virol. 47: 233-235.

Ecker, J.R. and R. Hyman. 1981. Varicella zoster virus vaccine DNA differs from the parental virus DNA. J. Virol. 40: 314-318.

Edson, C.M., B.A. Holser, and D.J. Waters. 1987. Varicella-zoster virus gpI and herpes simplex virus gE: phosphorylation and Fc binding. Virol. 161: 599-602.

Ellis, R.W., P.M. Keller, R.S. Lowe, and R.A. Zivin. 1985. Use of a bacterial expression vector to map the varicella-

zoster virus major glycoprotein gene, gC. J. Virol. 53: 81-88.

Everett, R.D.. 1986. The products of the herpes simplex type 1 (HSV-1) immediate early genes 1,2, and 3 can activate HSV-1 gene expression in trans. J. Gen. Virol. 67: 2507-2513.

Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6-13.

Feldman, S. and Epp E. 1979. Detection of viremia during incubation of varicella. J. Pediatr. 94: 746-748.

Felser, J.M., S.E. Straus, and J.M. Ostrove. 1987. Varicella-zoster virus complements herpes simplex virus type 1 temperature-sensitive mutants. J. Virol. 61: 225-228.

Felser, J., P.R. Kinchington, G. Inchauspe, S.E. Straus, and J.M. Ostrove. 1988. Cell lines containing varicella-zoster virus open reading frame 62 and expressing the "IE" 175 protein complement ICP4 mutants of herpes simplex virus type 1. J. Virol. 62: 2076-2082.

Fenner, F. 1948. The pathogenisis of the acute exanthums: An interpretation based on experimental investigations with

mousepox (infectious ectromelia of mice). Lancet 2: 915-920.

Fleisher, G., W. Henry, M. McSorley, A. Arbeter, and S. Plotkin. 1981. Life-threatening complications of varicella. Am. J. Dis. Child. 135: 896-899.

Frink, R.J., R. Eisenberg, G. Cohen, and E.K. Wagner. 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. 45:634-647.

Gelb, L.D., W.J. Wellinghoff, J.H. Martin, J.J. Huang. 1981. Varicella-zoster virus fails to induce immunoglobulin G Fc receptors in human cells. Proc. Soc. Biol. Med. 168: 228-232.

Gelb, L.D.. 1990. Varicella-Zoster Virus, p 2011-2054. In Fields (ed.) Virology second edition. Raven Press. Ltd.

Gilden, D.H., A. Vafai, Y. Shtram, Y. Becjer, M. Devlin, and M. Wellish. 1983. Varicella-zoster virus DNA in human sensory ganglia. Nature 306: 478-480.

Glisin, V., R. Crkvenjakov, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochem. 13: 2633-2637.

Godowski, P.J., D.M. Knipe. 1983. Mutations in the DNA binding protein gene of herpes simplex virus type 1 result in increased levels of viral gene expression. J. Virol. 47: 478-486.

Gorman, C.J., Moffat, L.F., and H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyl transferase in mammialian cells. Mol. Cell. Biol. 2: 1044-1051.

Graham, F.L. and A.J. Van Der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virol. 52: 456-467.

Grose, C., D.M. Perrootta, P.A. Brunell, and G.C. Smith. 1979. Cell-free varicella-zoster virus in cultured human melanoma cells. J. Gen. Virol. 43: 15-27.

Guess, H.A.. 1986. Population-based studies of varicella complications. Pediatr. 78: 723-727.

Hanahan, D. 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166: 557-580.

Harper, D.R. and H.O. Kangro. 1990. Lipoproteins of varicella-zoster virus. J. Gen. Vir. 71: 459-463.

Hayakawa, Y. and R. W. Hyman. 1987. Isomerization of the U_L region of varicella-zoster virus DNA. Vir. Res. 8: 25-31.

Hogan, E.L. and M.R. Krigman. 1973. Herpes zoster myelitis: evidence for viral invasion of spinal cord. Arch. Neurol. 29: 309-13.

Homa, F.L., T.M. Otal, J.C. Glorioso, and M. Levine. 1986a. Transcriptional control signals of a herpes simplex virus type 1 late (γ2) gene lie within bases -34 to +124 relative to the 5'terminus of the mRNA. Mol. Cell. Biol. 6: 3652-3666.

Homa, F. L., D. J. M. Purifoy, J. C. Glorioso and M. Levine. 1986b. Molecular basis of the glycoprotein C negative phenotypes of herpes simplex type 1 mutants selected with a virus neutralizing monoclonal antibody. J. Virol. **58**: 281-289.

Homa, F.L., J.C. Glorioso, and M. Levine. 1988. A specific 15-bp TATA box promoter element is required for expression of a herpes simplex virus type 1 late gene. Genes Dev. 2: 40-53.

Honess, R.W. and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14: 8-19.

Hope-Simpson, R.E. 1965. The nature of herpes zoster: a long-term study and a new hypothesis. Proc. Soc. Med. 58: 9-20.

Horikoshi, M., C.K. Wang, H. Fujii, J.A. Cromlish, P.A. Weil, and R.G. Roeder. 1989. Cloning and structure of a yeast gene encoding a general transcription initiation factor TFIID that binds to the TATA box. Nature 341: 299-303.

Hyman, R.W., J.R. Ecker, and R.B. Tenser. 1983. Varicella-zoster virus RNA in human trigeminal ganglia. Lancet 2: 814-816.

Inchauspe, G., S. Nagpal, and J.M. Ostrove. 1989a. Mapping of two varicella-zoster virus-encoded genes that activate the expression of viral early and late genes. Virol. 173: 700-709.

Inchauspe, G. and J.M. Ostrove. 1989b. Differential regulation by varicella zoster virus (VZV) and herpes simplex virus type-1 trans-activating genes. Virol. 173: 710-714.

Inoue, T. and T.R. Cech. 1985. Secondary structure of the circular form of the Tetrahymena rRNA intervening sequence: a technique for RNA structure analysis using chemical probes

and reverse transcriptase. Proc. Natl. Acad. Sci. USA 82: 648-652.

Johnson, D.C., M.C. Frame, M.W. Ligas, A.M. Cross, N.D. Stow. 1988. Herpes Simplex Virus Immunoglobin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. 62: 1347-1354.

Johnson, P.F. and S.L. McKnight. 1989. Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. 58: 799-839.

Johnson, R. and P.E. Milbourne. 1970. Central nervous system manifestations of chickenpox. Can. Med. Assoc. J. 102: 831-834.

Jones, K.A. and R. Tjian. 1985a. Sp1 binds to promoter sequences and activates herpes simplex virus 'immediate early' gene transcription *in vitro*. Nature 317: 179-185.

Jones, K.A., K.R. Yamamoto, and R. Tjian. 1985b. Two distinct transcription factors bind to the thymidine kinase promoter *in vitro*. Cell 42: 559-572.

Kadonaga, J.T., K.R. Carner, F.R. Masiarz, and R. Tjian. 1987. Isolation of cDNA encoding transcription factor Sp1

and functional analysis of the DNA binding domain. Cell 51: 1079-1090.

Kato, T., Kitamura K., Hayakawa, Y., M. Takahashi, A. Kojima, S. Sato,and K. Yamanishi. 1989. Transcription mapping of glycoprotein I (gpI) and gp IV of varicella zoster virus and immunological analysis of the gpI produced in cels infected with the recombinant vaccinia virus. Microbiol. Immunol. 33: 299-312.

Keller, P.M., B.J. Neff, and R.W. Ellis. 1984. Three major glycoprotein genes of varicella-zoster virus whose products have neutralization epitopes. J. Virol. 52: 293-297.

Keller, P.M., A.J. Davison, R.S. Lowe, C.D. Bennett, and R.W. Ellis. 1986. Identification and structure of the gene encoding gpII, a major glycoprotein of varicella-zoster virus. Virol. 152: 181-191.

Keller, P.M., A.J. Davison, R.S. Lowe, M.W. Rieman, and R.W. Ellis. 1987. Identification and sequence of the gene encoding gpIII, a major glycoprotein of varicella-zoster virus. Virol. 157: 526-533.

Kinchington, P.R., J. Remenick, J.M. Ostrove, S.E. Straus, W.T. Ruyechan, and J. Hay. 1986. Putative glycoprotein gene of varicella zoster virus with variable copy numbers of a 42

base pair repeat sequence has homology to herpes simplex virus glycoprotein C. J. Virol. 59: 660-668.

Kinchington, P.R., G. Inchauspe, J.H. Subak-Sharpe, F. Robey, J. Hay, W.T. Ruyechan. 1988. Identification and characterization of a varicella-zoster virus DNA-binding protein using antiser directed against a predicted synthetic oligopeptide. J. Virol. 62: 802-809.

Kinchington, P.R., P. Ling, M.N. Pensiero, W.T. Ruyechan, B.Moss, and J. Hay. 1990. The glycoprotein products (gpV) of varicella zoster virus gene 14 and their defective accumulation in a live attenuated strain (Oka). J. Virol. Submitted for publication.

Kristie, T.M., and B. Roizman. 1984. Separation of sequences defining basal expression from those conferring α gene recognition within the regulatory domains of herpes simplex virus 1 α genes. Proc. Natl Acad. Sci. USA 81: 4065-4069.

Kristie, T.M., and B. Roizman. 1986. The binding site of the major regulatory protein α4 specifically associated with the promoter regulatory domains of α genes of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA 84: 71-75.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lipschutz, B. 1921. Untersuchungen uber die Atiologie der Krankheiten der Herpesgruppr (herpes zoster, herpes genitalis, herpes febrilis). Arch Dermatol 136: 428-482.

Longnecker, R., S. Chatterjee, R.J. Whitley, and B. Roizman. 1987. Identification of a herpes simplex virys glycoprotein gene within a gene cluster dispensible for growth in cell culture. Proc. Natl. Acad. Sci. 84: 4303-4307.

Mackem, S. and B. Roizman. 1982a. Differentiation between α promoter and regulator regions of herpes simplex virus I: the functional domains and sequence of a movable α regulator. Proc. Natl. Acad. Sci. USA 79: 4917-4921.

Mackem, S. and B. Roizman. 1982b. Structural features of the α gene 4,0,and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. 44: 939-949.

Maguire, H.F.and R.W. Hyman. 1986. Polyadenylated, cytoplasmic transcripts of varicella-zoster virus. Intervirol. 26: 181-191.

Manniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning; a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Maniatis, T., S. Goodbourn, and J.A, Fischer. 1987. Regulation of inducible and tissue-specific gene expression. Science 236: 1237-1244.

McCarthy A.M., L. McMahan, and P A. Schaffer. 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. 63: 18-27.

McCormick, W.F., R.L. Rodnitzky, S.S. Schochet Jr, and A.P. McKee. 1969. Varicella-zoster encephalomyelitis: a morphologic and virologic study. Arch. Neurol. 21:559-70.

McDevitt, M.A., M.J. Imperiale, H. Ali, and J.R. Nevins. 1984. Requirement of a downstream sequence for generation of a poly(A) addition site. Cell 37: 993-999.

McGeoch, D.J., Dolan, A., S. Donald, and F.J. Rixon. 1985. Sequence derermination and genetic content of the short unique region of the genome of herpes simplex virus type 1. J. Mol. Cell Bio. 181: 1-13.

McGeoch, D.J., A. Dolan, A., S. Donald, D.H.K. Brauer. 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nuc. Acids Res. 14: 1727-1745.

McGeoch, D.J., M.A. Dalrymple, A.J. Davison, A. Dolan, M.C. Frame, D. McNab, L.J. Perry, J.E. Scott, and P. Taylor. 1988. The complete DNA Sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69: 1531-1574.

McGeoch, D.J. 1989. The genomes of the human herpesviruses: Contents, relationships, and evolution. Ann. Rev. Microbiol. 43: 235-265.

McGregor, R.M.. 1957. Herpes zoster, chickenpox, and cancer in general practice. Br. Med. J. 1: 84-87.

McKee, T.A., G.H. Disney, R.D. Everett, and C.M. Preston. 1990. Control of expression of the varicella zoster virus major immediate early gene. J. Gen. Virol. 71: 897-906.

McKnight, J.L.C., T.M. Kristie, B. Roizman. 1987. The binding of the virion protein mediating α gene induction in herpes simplex virus 1 infected cells to its cis site requires cellular proteins. Proc. Natl. Acad. Sci. USA 84: 7061-7065.

McKnight, S.L., E.R. Gavis, and R.Kingsbury. 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell 25:385-398.

McKnight, S.L.. 1982a. Functional relationships between transcriptional control signals of the thymidine kinase gene of herpes simplex virus. Cell 31: 355-365.

McKnight, S.L. and R. Kingsbury. 1982b. Transcriptional control signals of a eukaryotic protein coding gene. Science 217: 316-324.

McKnight, S.L., R.C. Kingsbury, A. Spence, and M. Smith. 1984. The distal transcription signals of the herpesvirus tk gene share a common hexanucleotide sequence. Cell 37: 253-262.

McKnight, S., and R. Tjian. 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell 46: 795-805.

McLauchlan, J., D. Gaffney, J. L. Whitton, and J. B. Clements. 1985. The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3' termini. Nuc. Acids Res. 13: 1347-1368.

Michael, N., D. Spector, P. Mavromara-Nazos, T.M. Kristie, and B. Roizman. 1988. The DNA binding properties of the major regulatory protein α4 of herpes simplex viruses. Science 239: 1531-1534.

Mitchell, P.J., and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science. 245: 371-378.

Namazue J., H. Campo-Vera, K. Kitamura, T. Okuno, and N. Yamanishi. 1985. Processing of virus-specific glycoproteins of varicella-zoster virus. Virol. 143:252-259.

Nevins, J.R.. 1983. The pathway of eukaryotic mRNA formation. Ann. Rev. Biochem. 52: 441-66.

Nermut, M.V. and A.C. Steven. 1987. Animal Virus Structure. In Prospectives in Medical Virology volume 3 (ed). Elsevier Press. Ltd.

Ogata, M. and S. Shigeta. 1979. Appearance of immunoglobulin G Fc receptor in cultured human cells infected with varicella-zoster virus. Infect. Immun. 26: 770-774.

O'Hare, P. and G.S. Hayward. 1985. Evidence for a direct role for both the 175,000 and 110,000 molecular weight

immediate early proteins of herpes simplex virus in the transactivation of delayed early promoters. J. Virol. 53: 751-760.

Okuno, T., K. Yamanishi, K. Shiraki, and M. Takahashi. 1983. Synthesis and processing of glycoproteins of varicella-zoster virus (VZV) as studied with monoclonal antibodies to VZV antigens. Virol. 129: 357-368.

Ostrove, J. M., W. Reinhold, C.M. Fan, S. Zorn, J. Hay and S.E, Straus. 1985. Transcriptional mapping of the varicella zoster virus genome. J. Virol. 56: 600-606.

Paryani, S.G. and A.M. Arvin. 1986. Intrauterine infection with varicella-zoster virus after maternal varicella. N. Engl. J. Med. 314: 1542-6.

Pitel, P.A., K.L. McCormick, E. Fitzgerald, and J.M. Orson. 1980. Subclinical hepatic changes in varicella infection. Pediatr. 65: 631-3.

Preblud, S.R., W.A. Orenstein, and K.J. Bart. 1984. Varcella: clinical manifestations, epidemiology, and health impact in children. Pediatr. Infect. Dis. 3: 505-509.

Preston, C.M., M.C. Frame, M.E.M. Campbell. 1988. A complex formed between cell components and an HSV structural

polypeptide binds to a viral immediate early regulatory DNA sequence. Cell 52: 425-434.

Reichman, R.C.. 1978. Neurologic complications of varicella-zoster infections. Ann. Intern. Med. 89: 379-80.

Reinhold, W.C., S.E. Straus, and J.M. Ostrove. 1988. Directionality and further mapping of varicella zoster virus transcripts. Vir. Res. 9: 249-261.

Rice, S.A., D.M. Knipe. 1988. Gene-specific trans-activation by the herpes simplex virus type 1 alpha protein ICP27. J. Virol. 62: 3814-3823.

Rixon F.J. and D.J. McGeoch. 1985. Detailed analysis of the mRNAs mapping in the short unique region of herpes simplex virus type 1. Nuc. Acids Res. 13: 953-973.

Roberts, M.S., A. Boundy, P. O'Hare, M.C. Pizzorno, D.M. Ciufo, and G.S. Hayward. 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. 62: 4307-4320.

Roizman, B., L.E. Carmicheal, F. Deinhart, G. de-The, A.J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi,

K. Wolf. 1981. Herpesviridae. Definition, privisional nomenclature and taxonomy. Intervirol. 16: 201-218.

Roizman, B., A.E. Sears. 1990. Herpes simplex viruses and their replication, p. 1795-1841. In Fields (ed.) Virology second edition. Raven Press. Ltd.

Ruckdeschel, J.C., S.C. Schimpff SC, A.C. Smyth AC, and M.R. Mardiney. 1977. Herpes zoster and impaired cell associated immunity to the varicella-zoster virus in patients with Hodgkin's disease. Am. J. Med. 62: 77-85

Ruyechan, W.T., P. Ling, P.R. Kinchington, and J. Hay. 1990. The correlation between varicella-zoster virus transcription and the sequence of the viral genome. In The Herpesviruses (ed) second edition. Raven Press. Ltd. (in press)

Sacks, R.W., C.C. Green, D.P. Ashman, and P.A. Schaffer. 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. 55: 796-805.

Sacks, W.R., and P.A. Schaffer. 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J. Virol. 61: 829-839.

Sanger, F., A.R. Coulson, B.G., Barrel, A.J.H. Smith, and B.A. Roe. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. 143: 161-178.

Santoro, C., N. Mermod, P.C. Andrews, and R. Tjian. 1988. A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature 334: 218-224.

Sawyer, M.H., J.M. Ostrove, J.M. Felser, and S.E. Straus. 1986. Mapping of the varicella zoster virus deoxypyrimidine kinase gene and preliminary identification of its transcript. Virol. 149: 1-9.

Schmidt, M.C., C.C. Kao, R. Pei, and A.J. Berk. 1989. Yeast TATA-box transcription factor gene. Proc. Natl. Acad. Sci. USA 86: 7785-7789.

Sekulovich, R.E., K. Leary,, and R.M. Sandri-Goldin. 1988. The herpes simplex type 1 alpha protein ICP27 can act as a trans-repressor and trans-activator in combination with ICP4 and ICP0. J. Virol. 61: 1092-1097.

Shapira M., F.L. Homa, J.C. Glorioso, and M. Levine. 1987. Regulation of the herpes virus type 1 late (g_2) glycoprotein C gene; sequences between base pairs -34 to +29 control transient expression and respnsiveness to transactivation by

the products of the immediate early (a) 4 and 0 genes. Nuc. Acids Res. 15: 3097-3111.

Shiraki, K. and R.W. Hyman. 1987. The immediate early proteins of varicella-zoster virus. Virol. 156:423-426.

Southern, E.M. 1975. Detection of specific sequences amoung DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Stevens, J.G., E.K. Wagner, G.B. Devi-Rao, M.L.Cook, and L.T. Feldman. 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science. 235: 1056-9.

Stow, N.D. and E.C. Stow. 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide $V_{mw}110$. J. Gen. Virol. 67: 2571-2587.

Straus, S.E.,H.S. Aulakh, W.T, Reuychan, J. Hay, T.A. Casey, G.F. Vande Woude, J. Owens, and H.A. Smith. 1981. Structures of varicella-zoster virus DNA. J. Virol. 40: 516-525.

Straus, S.E., J. Owens, W.T. Ruyechan, H.E. Takiff, T.A. Casey, G.F. Vande Woude, and J. Hay. 1982. Molecular

cloning and physical mapping of varicella-zoster virus DNA. Proc. Natl. Acad. Sci. USA 79: 993-997.

Straus, S.E., J. Hay, H. Smith, and J. Owens. 1983. Genome differences among varicella-zoster virus isolates. J. Gen. Virol. 64: 1031-1041.

Straus, S.E., W. Reinhold, H.A. Smith. 1984. Endonuclease analysis of viral DNA from varicella and subsequent zoster infection in the same patient. N. Engl. J. Med. 311: 1362-1364.

Sturm, R.A., G. Das, W. Herr. 1988. The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. Gen. Dev. 2: 1582-1599.

Takahashi, M., Y. Okuno, Y. Otsuka, J. Osame, A. Takamizawa, T. Sasada, and T. Kubo. 1975. Development of a live attenuated varicella vaccine. Biken J. 18: 25-33.

Takahashi, M. 1984. Developement and characterization of a live varicella vaccine (Oka strain). Biken J. 27: 31-36.

Thompson, R., R.W. Honess, L. Taylor, J. Morran, and A.J. Davison. 1987. Varicella-zoster virus specifies a thymidylate synthetase. J. Gen. Virol. 68: 1449-1455.

Triezenberg, S.J., K.L. LaMarco, S.L. McKnight. 1988. Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes Dev. 2: 730-742.

Vafai, A., Z. Wroblewska, R. Mahalingam, G. Cabirac, M. Wellish, M. Cisco, and D. Gilden. 1988. Recognition of similar epitopes on varicella-zoster virus gpI and gpIV by monoclonol antibodies. J. Virol. 62: 2544-2551.

Von Bokey, J. 1909. Uber den atiologischen zusammenhang der varizellen mit gewissenfallen von herpes zoster. Wein klin Wchschr. 22: 1323-1327.

Watson, P.N. and R.J. Evans. 1986. Postherpetic neuralgia: a review. Arch. Neurol. 43: 836-40.

Weinstein, L. and R.H. Meade III. 1956. Respiratory manifestations of chickenpox. Arch. Inter. Med. 98: 91-99.

Weller, T.H. 1953. Serial propagation *in vitro* of agents producing inclusion bodies derived from varicella and herpes zoster. Proc. Soc. Exp. Biol. Med. 83: 340-346.

Weller, T.H. and A.H. Coons. 1954. Flourescence antibody studies with agents of varicella and herpes zoster propagated *in vitro*. Proc. Soc. Exp. Biol. Med. 86: 789-794.

Weller, T.H. and H.M. Witton. 1958. The etiologic agents of varicella and herpes zoster. Seriologic studies with the viruses as propagated *in vitro*. J. Exp. Med. 108: 869-890.

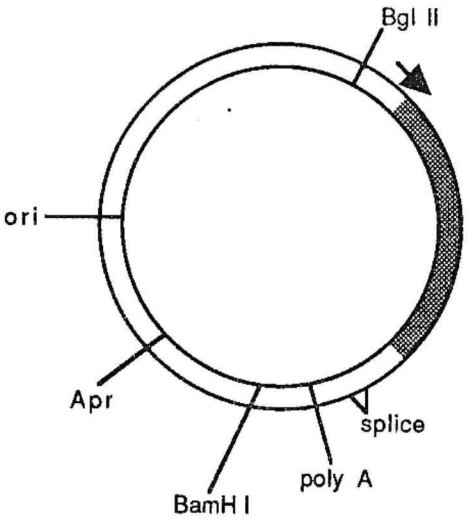
Appendix

The detailed cloning strategy for generation of recombinant DNA molecules specifically made for this study is listed in this appendix. Each clone is shown with a cloning strategy diagram and is accompanied by an ethidium bromide stained agarose gel of DNA derived from that clone.

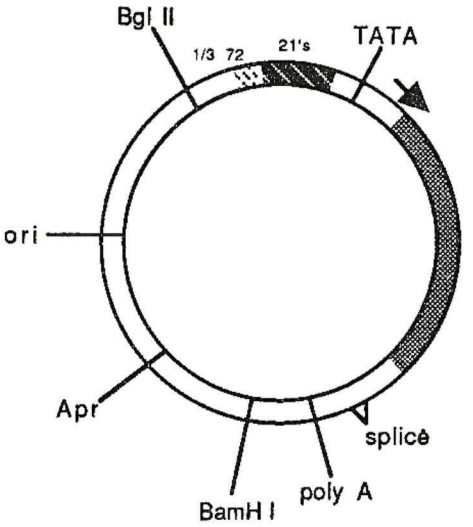
INDEX

clone	pg.
pCAT3M	A-1
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pSV2	A-1
5S1	A-2
3S2	A-3
5O4	A-4
S6	A-5
S2	A-5
O29	A-5
G4-2	A-6
G4-3	A-6
g4c	A-7
g4w	A-7
SF43	A-8
OF8a	A-8
SP1	A-9
OP1	A-9
PL1-7	A-10
vKIP5	A-11

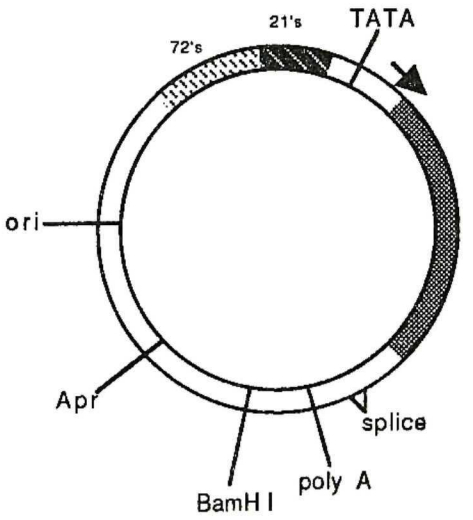
pCAT3M

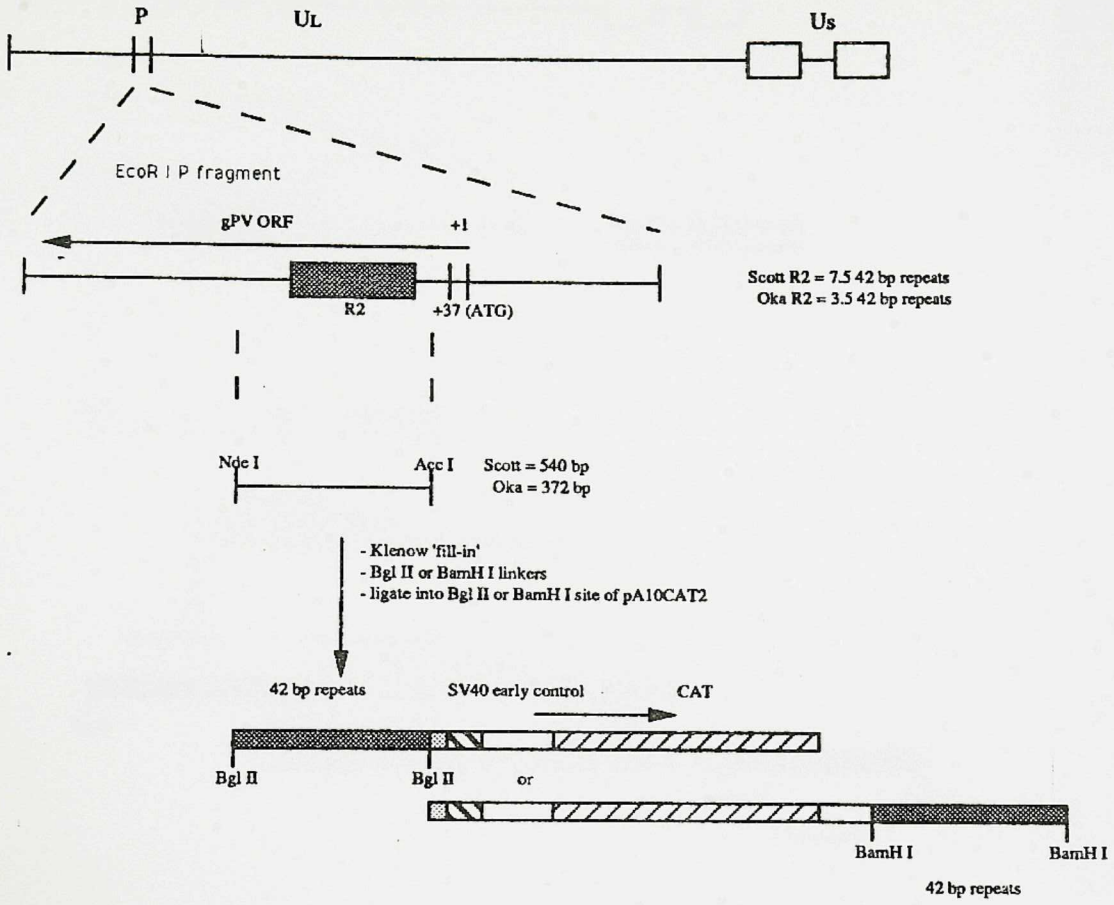


pA10CAT2



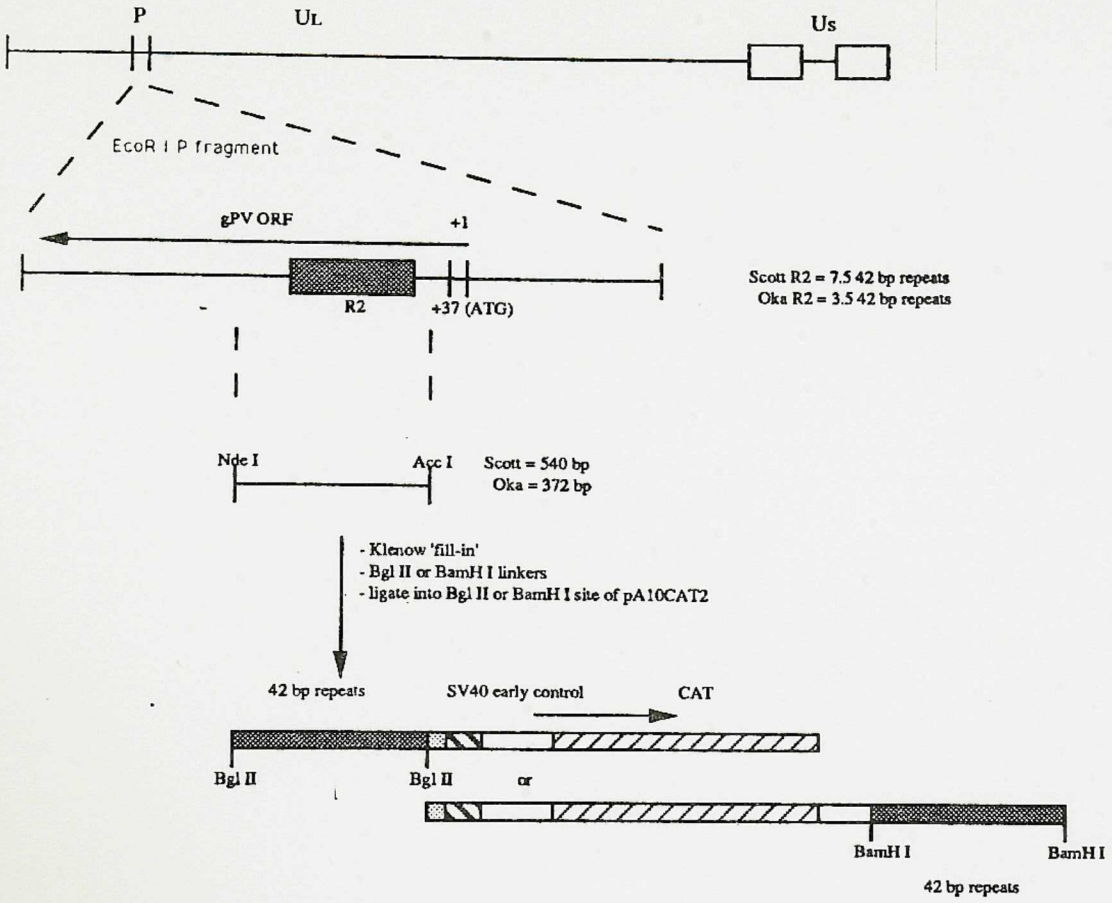
pSV2



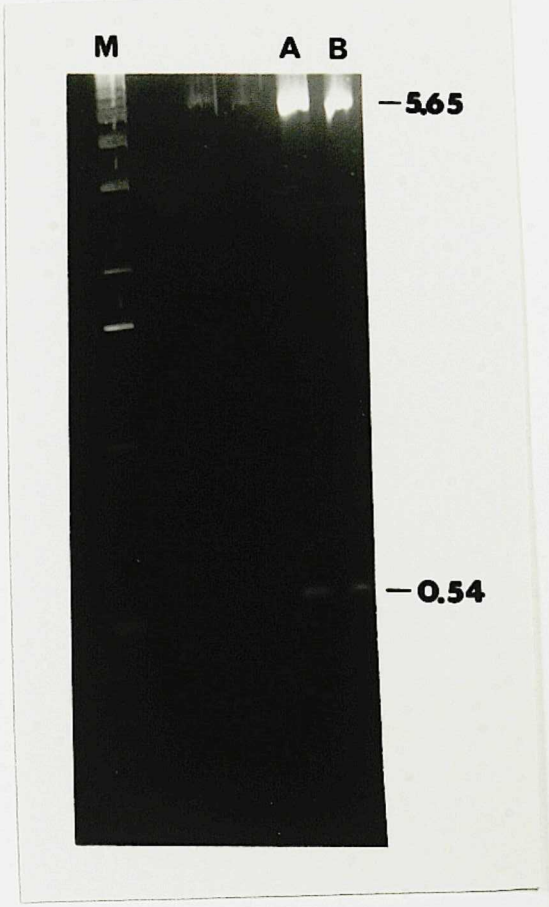


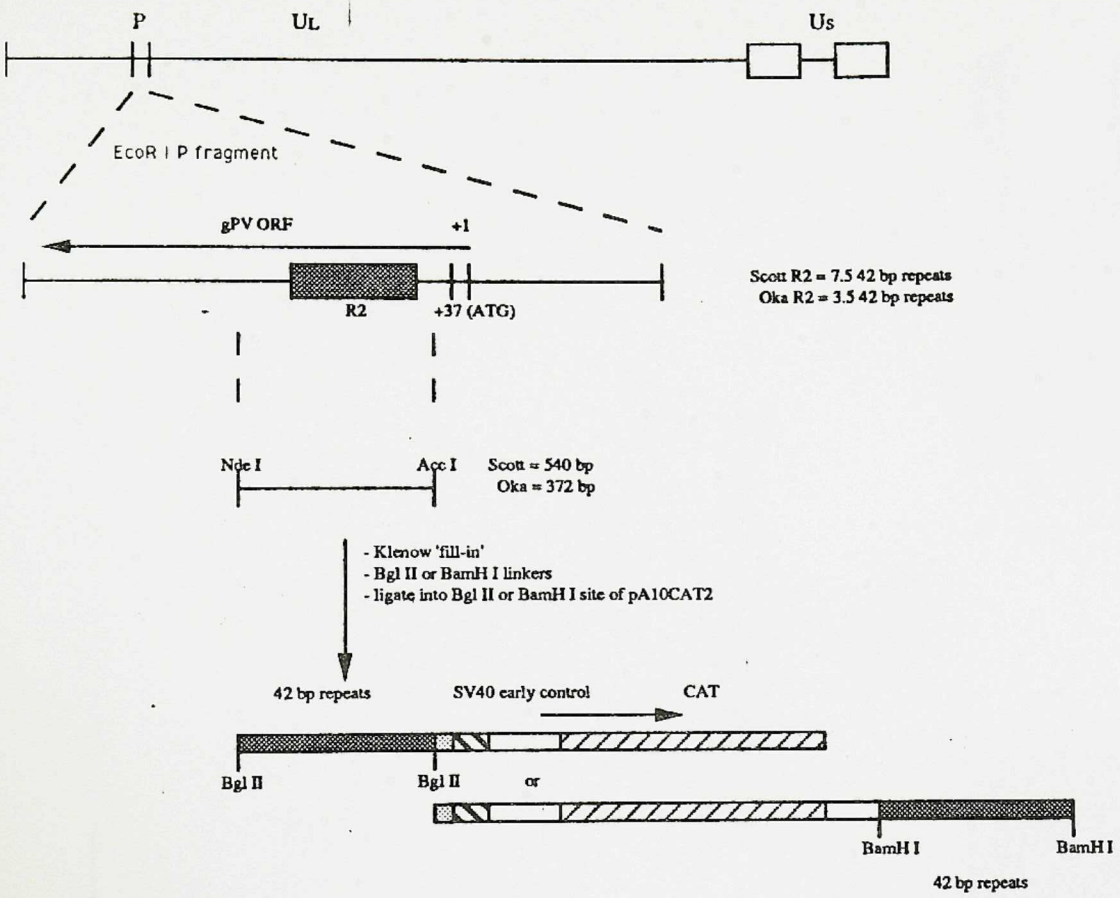
1.2% Agarose gel. Ethidium Bromode stained gel of DNA clones cut with restriction enzymes containing Scott 42bp repeats in the 5' position in pA10CAT2.
Lane A: 5S1 clone in the "sense' orientation cut with Bgl II
Lane B: 5S2 clone in the "anti-sense" orientation cut with Bgl II
M: 1kb ladder (BRL)
orientation was determined by sequencing the joints between VZV cloned sequences and pA10CAT2.



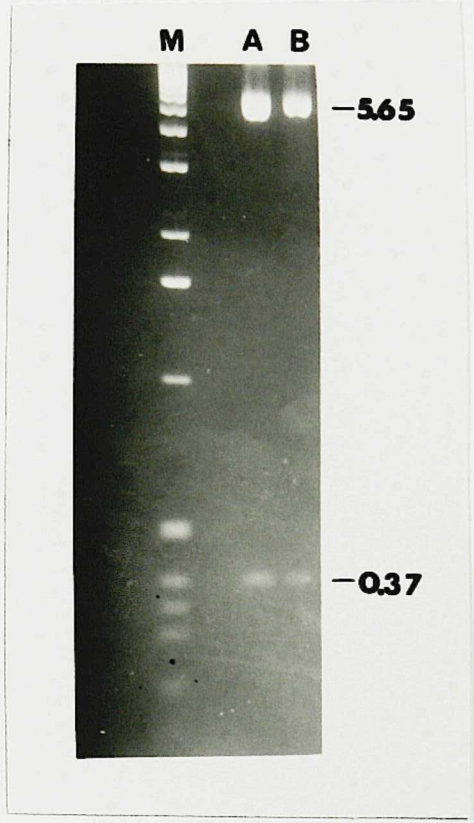


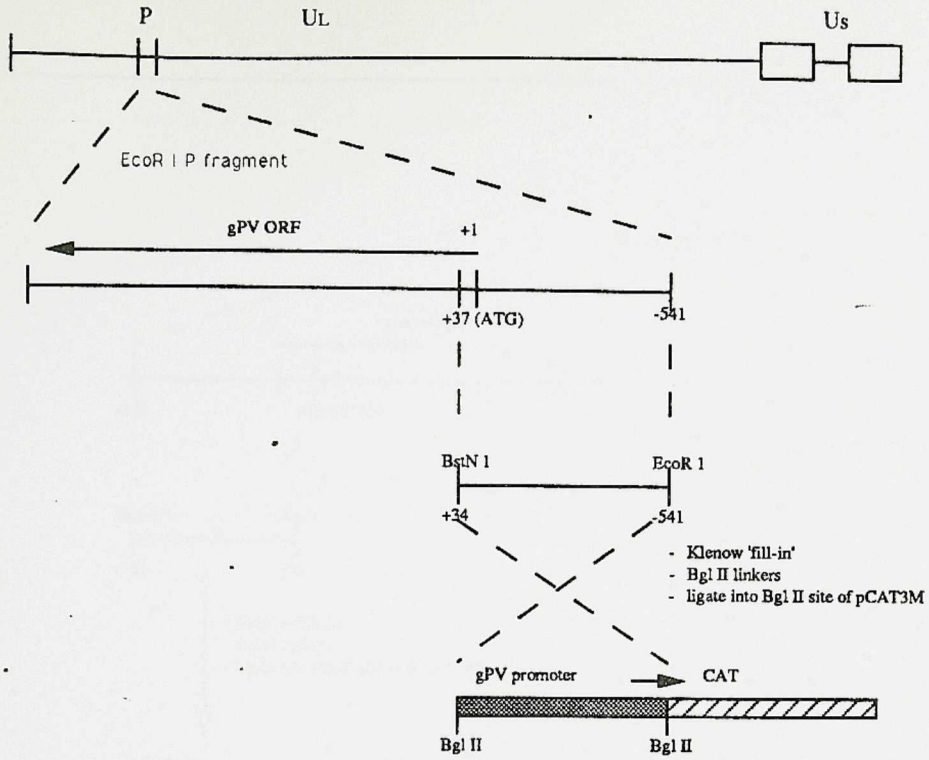
1.2% Agarose gel. Ethidium Bromode stained gel of DNA clones cut with restriction enzymes containing Scott 42bp repeats in the 3' position in pA10CAT2.
Lane A: 3S2 clone in the "sense' orientation cut with BamH I
Lane B: 3S5 clone in the "anti-sense" orientation cut with BamH I
M: 1kb ladder (BRL)
orientation was determined by sequencing the joints between VZV cloned sequences and pA10CAT2.





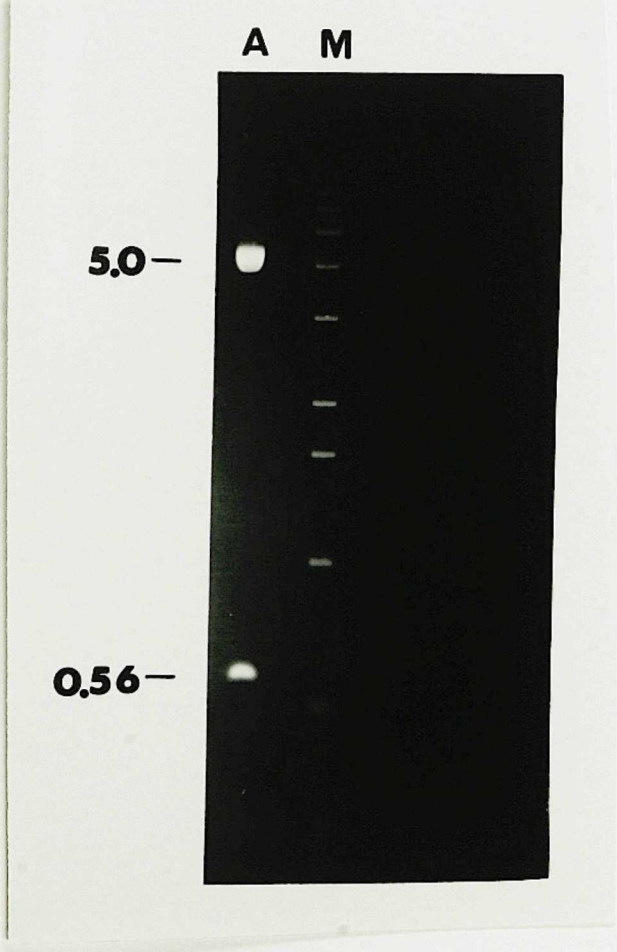
1.2% Agarose gel. Ethidium Bromode stained gel of DNA clones cut with restriction enzymes containing Oka 42bp repeats in the 5' position in pA10CAT2.
Lane A: 504 clone in the "sense' orientation cut with Bgl II
Lane B: 502 clone in the "anti-sense" orientation cut with Bgl II
M: 1kb ladder (BRL)
orientation was determined by sequencing the joints between VZV cloned sequences and pA10CAT2.





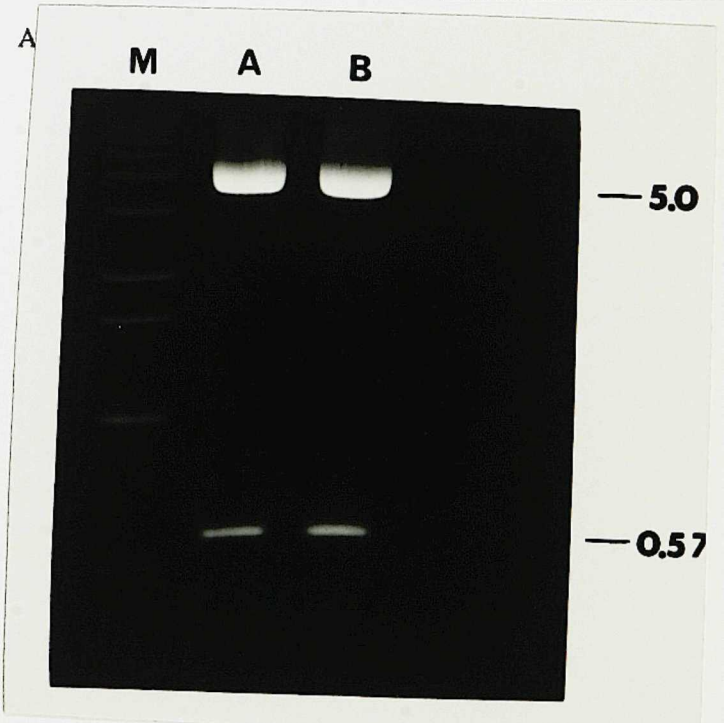
S6 = "sense"
S2 = "anti-sense"
O14 = "sense"
O29 ="anti-sense"

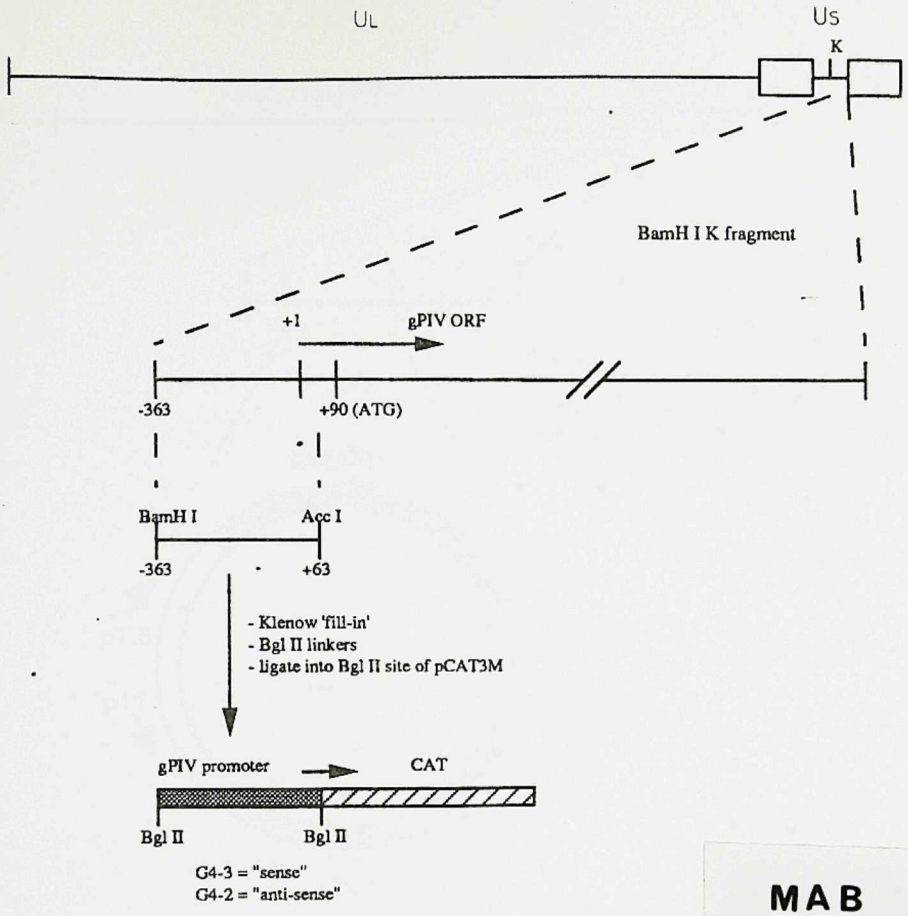
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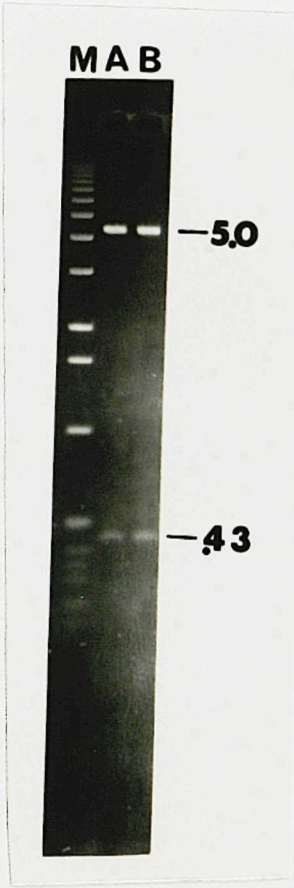


1.2% Agarose gels. Ethidium Bromide stained gels of Scott and Oka promoter/Cat fusion DNA plasmids.
Gel A-lane A: S6 cut with Bgl II
-lane B: S2 cut with Bgl II
Gel B-lane A: O29 cut with Bgl II
M: in both gels is a 1kb ladder (BRL)
Orientation was determined by sequencing the joint between VZV sequences and pCAT3M sequences.

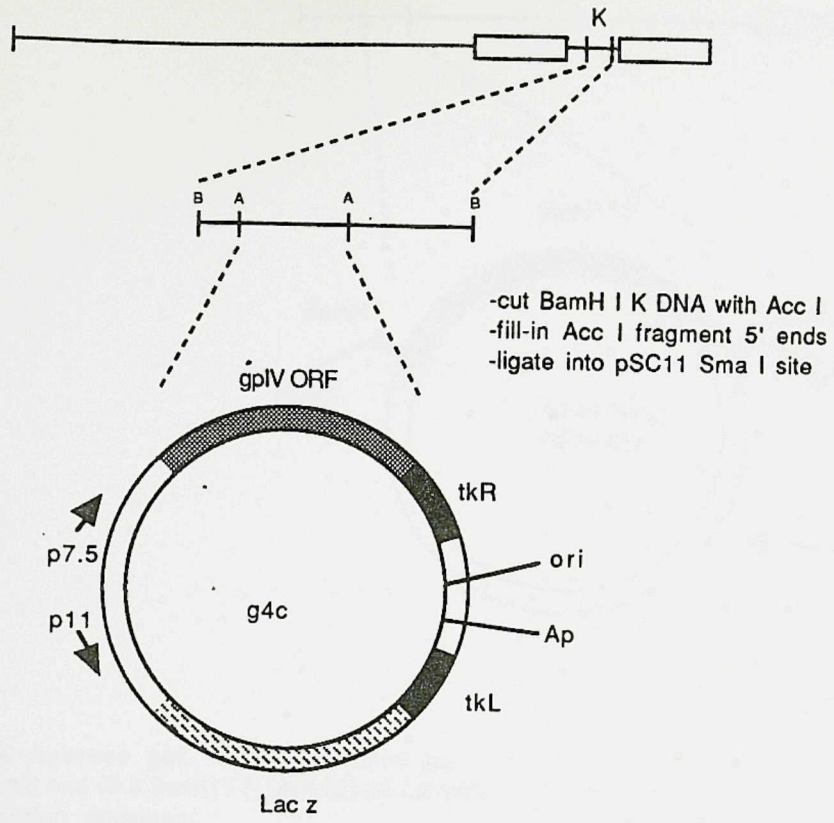
A







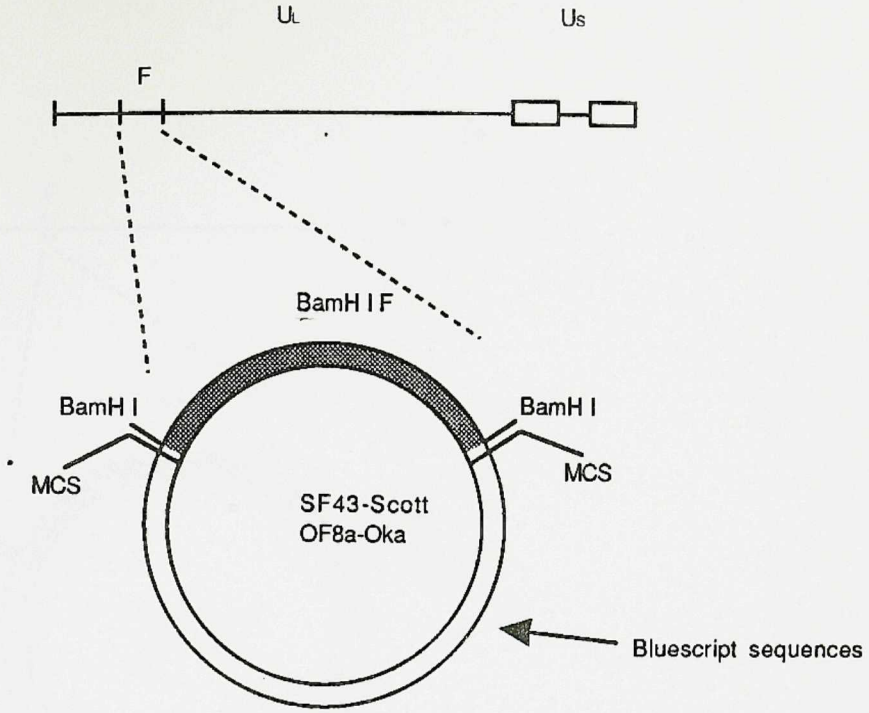
1.2% Agarose gel.. Ethidium Bromide stained gel of gpIV promoter/CAT fusion DNA plasmids.
Lane A: G4-3 clone cut with Bgl II
Lane B: G4-2 clone cut with Bgl II
M: 1kb ladder (BRL)
*orientation of clones was determined by sequencing the joint between VZV sequences and pCAT3M sequences.





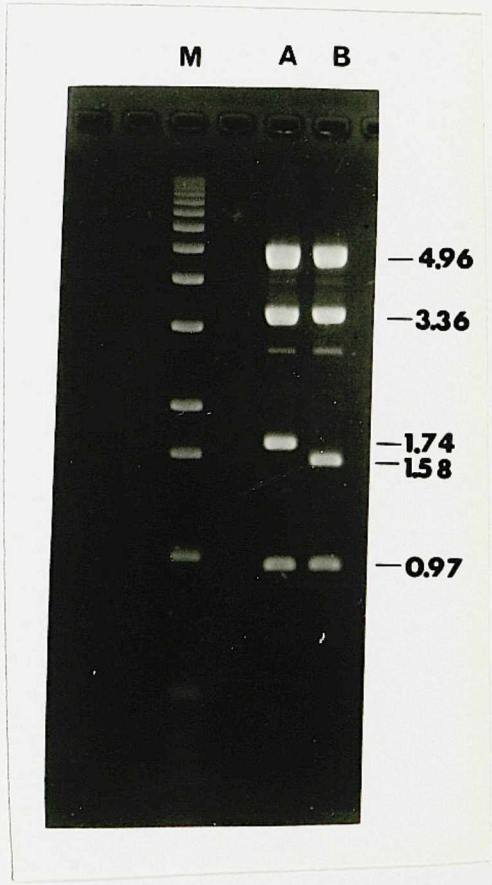
1% Agarose gel. Ethidium Bromide stained gel of plasmid construct DNA cut with restriction enzymes.
Lanes A and B: pSC11 DNA
Lanes C and D: g4c DNA
Lanes E and F: g4w DNA*
Lanes A,C,E cut with EcoR I and BamH I
Lanes B,D,F cut with BamH I and Nco I
M: 1kb ladder (BRL)

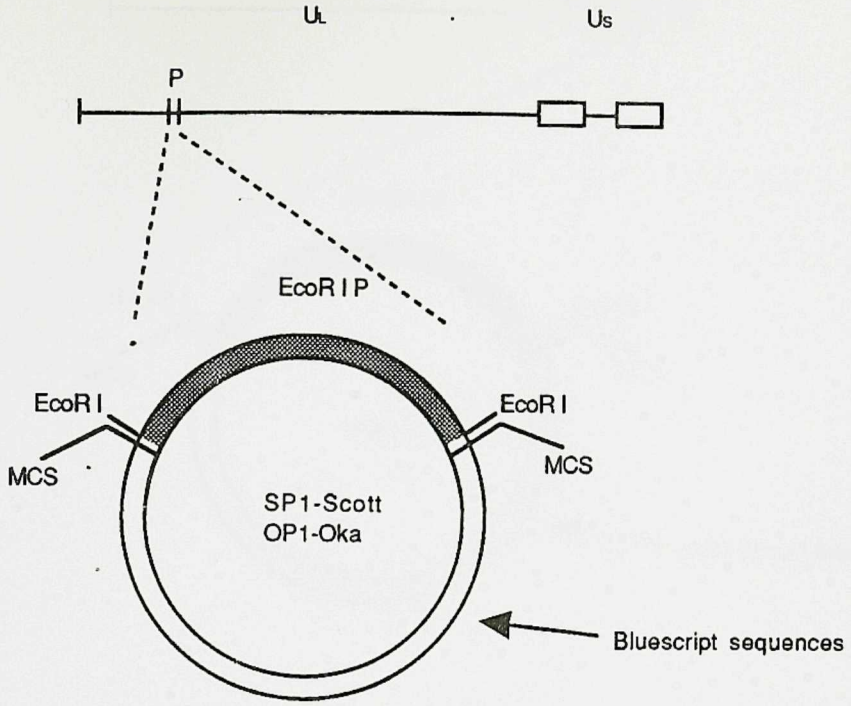
*g4w is a clone that would form if the gpIV ORF ligated into pSC11 in the opposite orientation to g4c, hence giving a different restriction map distiguishable from the desired clone as seen in the figure.



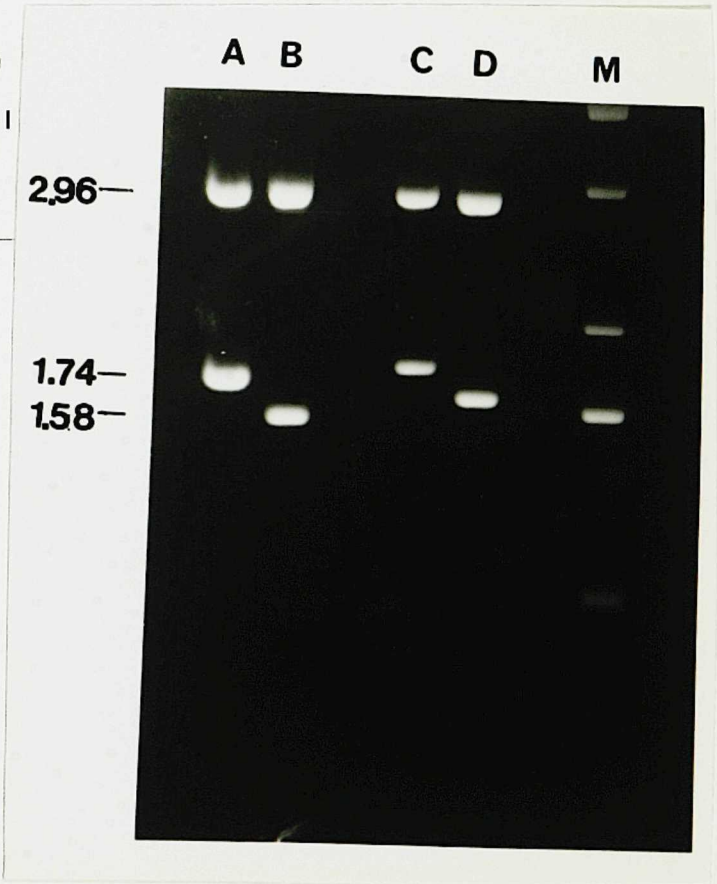
0.7% Agarose gel. Ethidium stained gel of Scott and Oka BamH I F DNA clones cut with restriction enzymes.
Lane A: Scott BamH I F clone (SF43) cut with EcoR I
Lane B: Oka BamH I F clone (OF8a) cut with EcoR I*
M: 1kb ladder (BRL)

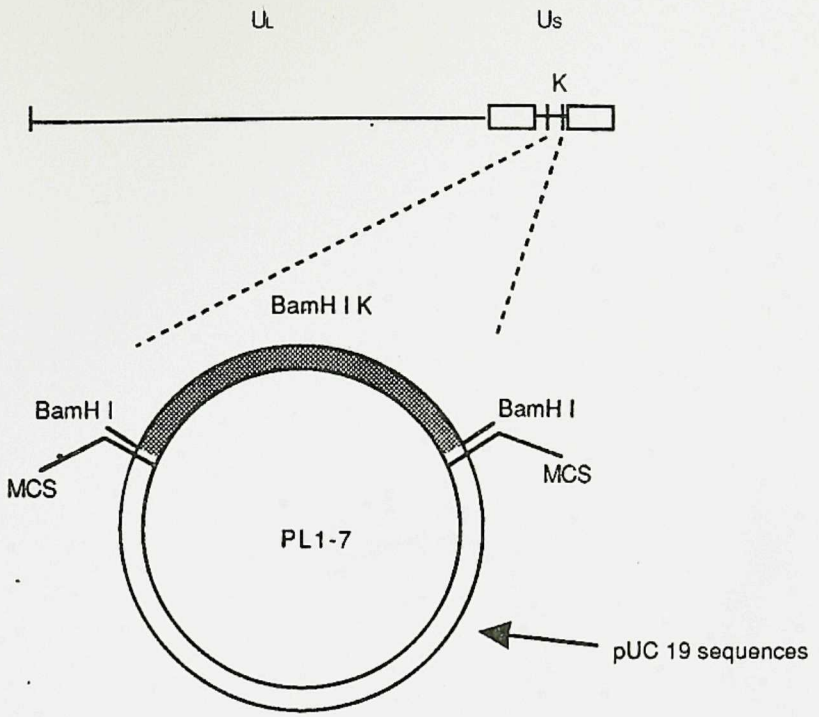
* notice the size difference in the EcoR I P fragments that are contained within the BamH I F fragment





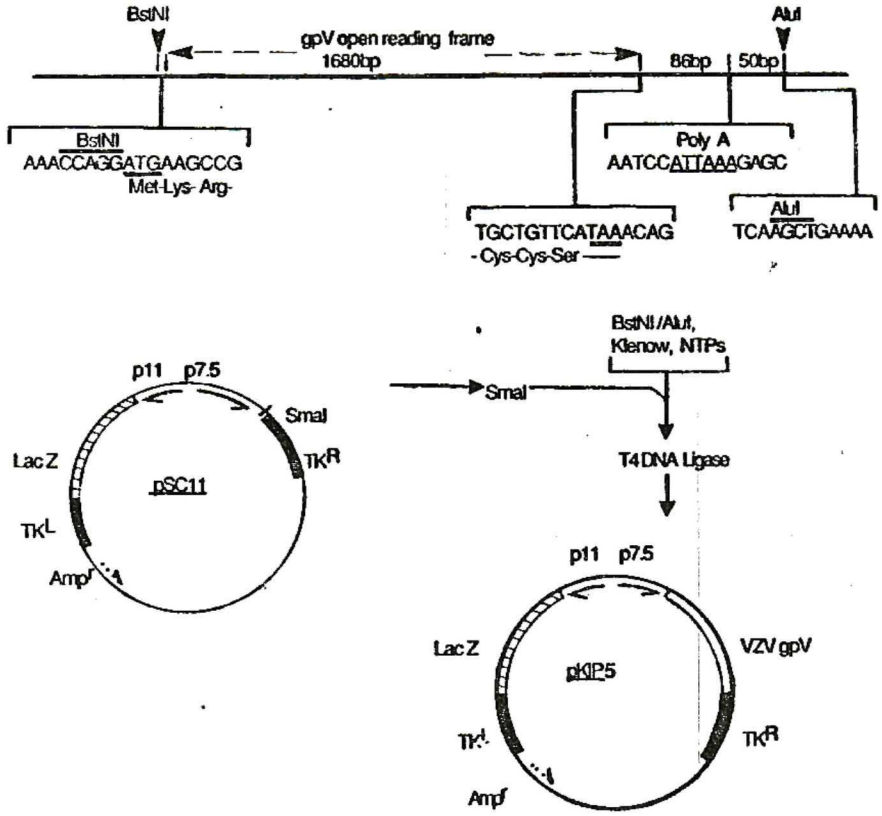
1% Agarose gel. Ethidium Bromide stained gel of Scott and Oka EcoR I P DNA clones cut with restriction enzymes.
LaneA: Scott EcoR I P clone (SP1) cut with EcoR I
Lane B: Oka EcoR I P clone (OP1) cut with EcoR I
Lanes C and D are clones containing the entire gpV ORF from Scott and Oka respectively, in pBluescript.
M: 1kb ladder(BRL)





1% Agarose gel. Ethidium stained gel of BamH I K DNA clones cut with restriction enzymes.
Lane B: PL1-7 clone cut with Bam H I
M: 1kb ladder (BRL)
*other lanes contain BamH I K clones in different vectors (lane C) or the vector in C, lane D. Lane A is an abberant clone.




BstNI
gpV open reading frame
1680bp
AluI
86bp
50bp
BstNI
AAACCAGGATGAAGCCG
Met-Lys-Arg-
Poly A
AATCCATTAAAGAGC
TGCTGTTCATAAACAG
-Cys-Cys-Ser—
AluI
TCAAGCTGAAAA
p11
p7.5
SmaI
LacZ
TK^R
pSC11
TK^L
Amp^r
BstNI/AluI,
Klenow, NTPs
SmaI
T4 DNA Ligase
p11
p7.5
LacZ
VZV gpV
pKIP5
TK^L
TK^R
Amp^r